

**The seasonal biology of the brachiopod *Liothyrella*  
*neozelanica* (Thomson, 1918) from Doubtful  
Sound, New Zealand.**

**Matthew John Baird**

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**Marine Science Department**

**University of Otago**

**Dunedin, New Zealand**

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# Abstract

*Liothyrella neozelanica* (Thomson 1918) (Subphylum: Rhynchonelliformea) is a temperate brachiopod that makes up ecologically important habitat in Doubtful Sound, New Zealand. From December 2010 to December 2011, the seasonal metabolism, seasonal biochemistry, reproductive cycle and annual growth of *L. neozelanica* were investigated. Findings enabled direct biological knowledge of a temperate New Zealand brachiopod, providing insight into how the genus *Liothyrella* has adapted to both temperate and polar environments. Furthermore, the findings enabled insight into how increasing sea temperatures at mid and high latitudes may affect brachiopods belonging to the genus *Liothyrella* over the next century, as a result of climate change. Seasonal ambient respiration rates of *L. neozelanica* ranged between 243.5 and 560  $\mu\text{g}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$  suggesting *L. neozelanica* actively adjusts its metabolism to changing surrounding environmental conditions. *L. neozelanica* showed evidence of thermal compensation to seasonal changes in ambient sea temperature and displayed a broad thermal tolerance; aerobically respiring at temperatures 5°C above the maximum ambient sea temperature in Doubtful Sound (18°C). Soluble protein was the major energy substrate utilised within the internal tissues of *L. neozelanica*; however lipid was found to occur in high levels within the gonads (46% gonad AFDM) suggesting it is stored during reproductive development. Gametogenic observations suggest that *L. neozelanica* spawns between July and September, although the occurrence of mature oocytes within female gonads throughout an annual period suggests *L. neozelanica* may undergo multiple spawning events annually. Spawning is likely cued to photoperiod and primary productivity. Growth by *L. neozelanica* was found to be highest in juveniles (8.4 - 8.9 mm  $\text{yr}^{-1}$ ). The absence of high numbers of juvenile *L. neozelanica* (< 10 mm length) in a population size-frequency distribution, suggests juvenile *L. neozelanica* may require a size-refuge to avoid early mortality. Annual growth cohorts suggest recruitment takes place annually. Biological comparisons between *L. neozelanica* and the Antarctic brachiopod *Liothyrella uva* (Broderip 1833) revealed *L. neozelanica* to have metabolic rates (89 – 110  $\mu\text{g}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$ ) 1.9 to 2.9 times higher than those exhibited by *L. uva* (38 – 46  $\mu\text{g}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$ ) after a  $Q_{10}$  correction was applied. Biochemical comparisons revealed both species utilise protein, carbohydrate and lipid in similar ways suggesting this may be a historic link between the two brachiopods. Both species showed evidence of spawning in spring which may occur to coincide with increasing sea temperatures and primary productivity. Growth comparisons revealed *L. neozelanica* to grow 3 to 5 times faster than *L. uva* despite reaching similar

maximal sizes ( $S_{\infty}$ (mm length) = 52.5 (*L. neozelanica*); 47.2 – 54.1 (*L. uva*)). *L. uva* was found to have a greater life-span compared to *L. neozelanica* (30 + years (*L. neozelanica*); 46 – 71 years (*L. uva*)). Based on the findings in this study it is suggested that *L. neozelanica* will be largely unaffected by warming sea temperatures resulting from climate change over the next century. In contrast, evidence suggests *L. neozelanica*'s polar sister species *Liothyrella uva* may face physiological (metabolism) and biological (growth and energetics) difficulties as sea temperatures increase.

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# CHAPTER 1

## GENERAL INTRODUCTION



Hall Arm, Doubtful Sound (January, 2011).

**“A journey of a thousand li (500 meters) starts with a single step”**

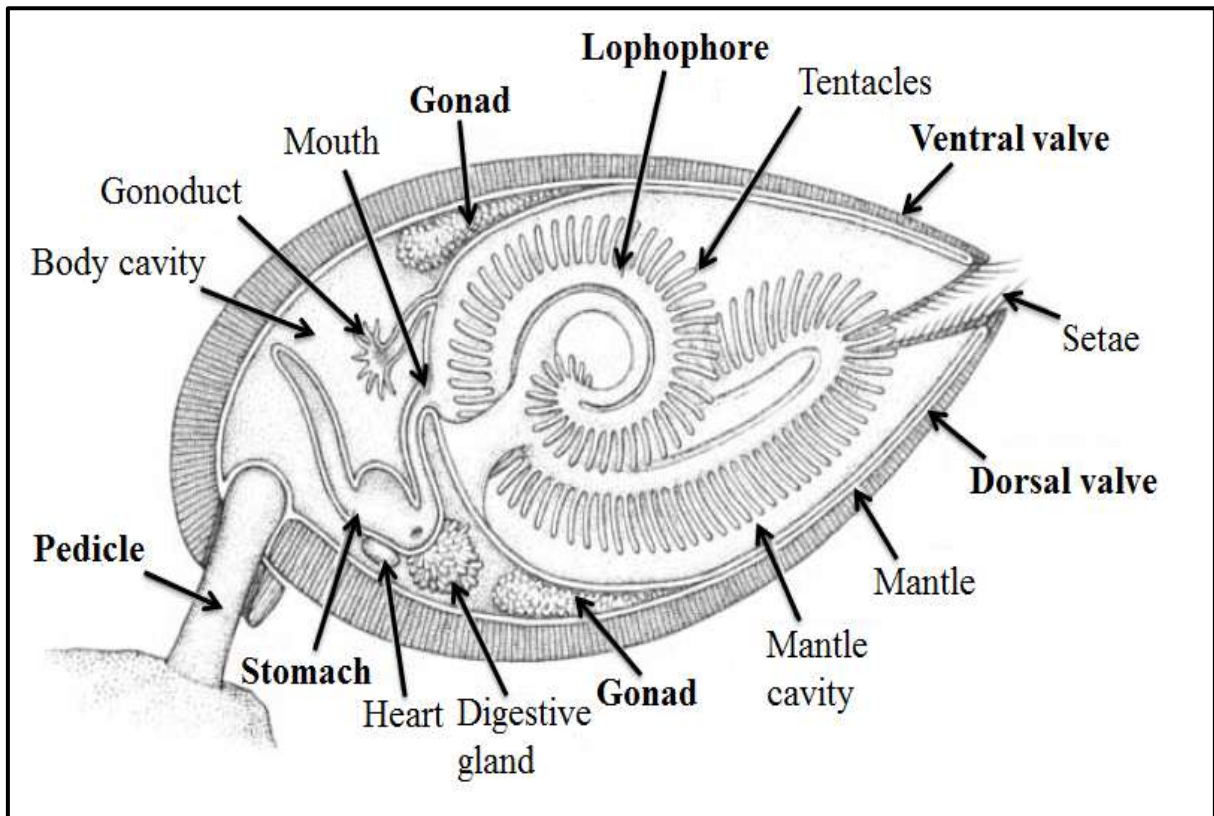
**(老子 (Lǎozǐ): 6<sup>th</sup>- 5<sup>th</sup> Century BC)**

## 1.1 Phylum Brachiopoda

Brachiopods (Phylum Brachiopoda) are suspension feeding invertebrates, characterised as marine lophophorates (Rudwick 1970; Rhodes and Thompson 1992; Emig 2009). They are classified into three subphyla; Linguliformea, Craniiformea and Rhynchonelliformea (Emig 2009). Linguliformea are characterised by organophosphatic shells and the development of a pedicle from the ventral mantle (MacFarlan *et al.* 2009). Craniiformea are characterised as having calcium carbonate shells and the absence of a pedicle; instead directly cementing their shells to substrate (MacFarlan *et al.* 2009). Rhynchonelliformea are characterised by the development of a pedicle from a segment of larvae during development (MacFarlan *et al.* 2009). Although diverse in appearance, brachiopods share a common simple physiology and anatomy that has enabled them to successfully colonise the oceans from littoral zones to abyssal waters (Richardson 1986; Emig 2009).

Brachiopods are comprised of two bilaterally symmetrical shells (dorsal and ventral) which house the interior mantle and organs (Fig. 1.1). The lophophore is the dominating organ within the mantle cavity and is used for feeding, respiration, excretion and in some cases for brooding juvenile larvae. Within the mantle two other prominent organs, the gonads and the stomach, sit alongside a heart, a pair of gonoducts and a digestive gland (Rudwick 1970; Richardson 1986; James *et al.* 1992). Brachiopods also possess a specific organ known as the pedicle. The pedicle is used to directly attach brachiopods to surrounding substrate or to anchor them within sediment. Although most living brachiopods are attached to hard substrate, they can use their pedicle to adjust themselves accordingly to surrounding conditions (Richardson 1986).

Brachiopods appear structurally simple however they can function in almost any marine environment (Richardson 1986). Consequently, brachiopods have existed over a vast period of time, surviving mass extinctions and rapid shifts in environmental conditions, reflecting the resilience of the phylum (Richardson 1981a; Rhodes and Thompson 1992).



**Figure 1.1** Generalised anatomy of a brachiopod dissected down the symmetrical plane (Adapted from Richardson 1986).

The evolutionary history of brachiopods spans over 500 million years (Rudwick 1970). Due to their vast existence and shells that are robust and composed of stable calcite, their remains are abundant in fossil-bearing rocks ranging from the Cambrian to the Cenozoic (Rudwick 1970; Richardson 1981a). Brachiopods were the dominant invertebrates of the oceans in both diversity and abundance throughout the Paleozoic and Mesozoic eras (Curry *et al.* 1989; Rhodes and Thompson 1992; Lee 2008). However, the majority of brachiopod species became extinct during the end of the Permian mass extinction episode (Thayer 1986; Pennington and Stricker 2001). Of the 5000 genera identified from fossil records only around 400 species from 100 genera remain today (Lee 2008; Zezina 2008; Emig 2009). Following the Permian/Triassic extinction the global plankton compositions changed, which in turn affected many brachiopod species which had evolved specialised feeding habits (Zezina 2008). As a result, many ecological niches that were once occupied by brachiopods were left unfilled. Bivalves with broad feeding capabilities began to occupy these niches and radiate throughout the world's oceans (Zezina 2008). Consequently brachiopods were 'outcompeted' by bivalves and have since been predominately confined to areas where competition between the two

phyla is low (Rhodes and Thompson 1992; Rhodes and Thompson 1993). High-energy, food-rich, near-shore habitats tend to be dominated by bivalves. In contrast brachiopods generally occur in low-energy, food-poor habitats such as caves, dark fiord walls, polar regions and abyssal waters (Rudwick 1970; Rhodes and Thompson 1992; Lee 2008). Low metabolic rates, linked with low energy demands have been essential in enabling brachiopods to occupy areas where bivalve competition is low (Richardson 1986; Grange and Singleton 1988; Rhodes and Thompson 1992).

Despite being restricted to specific areas, brachiopods have a wide latitudinal distribution, occupying a large range of substrates including rock, sand, shells and seaweeds. As a result, brachiopods make up a small but significant part of the sessile benthic fauna in areas where they occur (Rudwick 1970; Richardson 1981b; James *et al.* 1992). Due to the surviving brachiopods having a relatively unchanged form from their ancestors, they enable a rare opportunity to gain insight into the ecology, biology and environmental tolerances of this long-lived phylum.

Historically, the majority of brachiopod research has been confined to the areas of paleontology and geology as they occur extensively throughout the fossil record and have little or no commercial value (Richardson 1986; Pennington and Stricker 2001). In recent years, the distribution, ecology and general biology of brachiopods have received growing amounts of interest as they are often ecologically important constituents within relatively harsh environments. Due to their comparative simplicity brachiopods have been regarded as model invertebrates for understanding the required adaptations needed to survive in areas that are subjected to difficult environmental conditions (Peck *et al.* 1986; Peck *et al.* 1987; Lee 2008). The bulk of brachiopod research has been primarily concentrated in areas where large numbers occur in accessible environments (Richardson 1981b; James *et al.* 1991b; Lee *et al.* 2010). As a result, Antarctic and New Zealand brachiopods have received growing amounts of attention, particularly in regards to the genus *Liothyrella* (Wisely 1969; Peck *et al.* 1986; Rhodes and Thompson 1992; Peck *et al.* 1997; Lee *et al.* 2010; Lurman *et al.* 2010).

## 1.2 Genus: *Liothyrella* (Thomson, 1916)

The “short looped” brachiopod genus *Liothyrella* (Subphylum: Rhynchonelliformea, Family: Terebratulidae), has a wide latitudinal distribution with species occurring in the Pacific Ocean, Southern Indian Ocean, Atlantic Ocean and the Southern Ocean (Jackson 1918; Peck *et al.* 1986; Zvezina 2008; Emig 2009; Lee *et al.* 2010). Typically, *Liothyrella* brachiopods are characterised as achieving a relatively large size and as having broad outer hinge plates (Foster, 1974). Two species that have received considerable biological attention are the Antarctic species *Liothyrella uva* (Broderip 1833) and the temperate New Zealand species *Liothyrella neozelanica* (Thomson 1918) (Peck *et al.* 1986; Peck *et al.* 1987; Chuang 1994; Peck *et al.* 1997; Lee *et al.* 2010; Lurman *et al.* 2010).

*Liothyrella uva* occurs in the Southern Ocean at depths of five to 2150 meters (Dell 1972) (Fig. 1.2). Attached singly or in clumps to vertical and overhanging rocks, *L. uva* typically occurs around the South Orkney Islands, the Antarctic Peninsula and Peter I Island (Foster 1974). Substantial research describing seasonal changes in metabolism, biochemistry, feeding rates, growth and ontogenetic size has been conducted for *L. uva* (Peck *et al.* 1986; Peck *et al.* 1987; Peck and Holmes 1989a, b; Peck 1996; Peck *et al.* 1997; Lurman *et al.* 2010). The majority of this research focused on the seasonal respiration rates and biochemical components, providing insight into the metabolic scope and energy stores of *L. uva* over an annual period (Peck *et al.* 1987; Peck *et al.* 1997).

*L. uva* has been described as a key species which provides insights into Antarctic invertebrate adaptations (Peck *et al.* 1986). However, the majority of the metabolic and biochemical research on this brachiopod has compared it to other marine invertebrates, in particular molluscs, rather than to the closely related temperate brachiopod *L. neozelanica*. Furthermore, comparative work between Antarctic and temperate brachiopods has involved brachiopods from different genera (Peck *et al.* 1986; Peck *et al.* 1987). A recent paper describing the differences in mitochondria between *L. uva* and *L. neozelanica* is one of the few to directly compare *L. uva* with a temperate brachiopod from the same genus (Lurman *et al.* 2010). To fully quantify the adaptations that *L. uva* has made to its environment, it is necessary to compare findings from closely related temperate species. These comparisons will enable insights into how *L. uva* has specifically adapted its physiology and biology to suit the

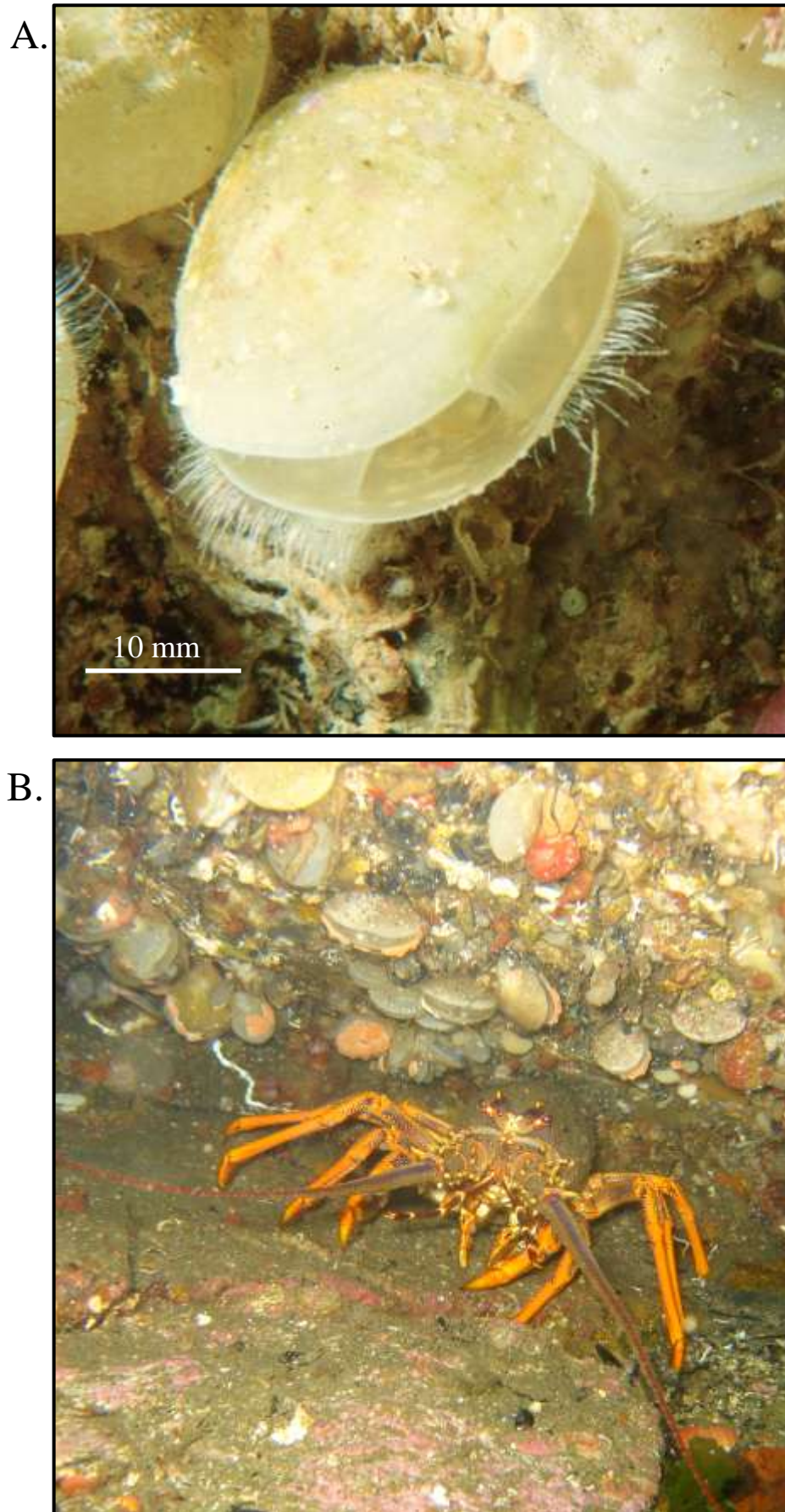
Southern Ocean environment and may provide clues into how the species may be affected by future environmental change.

*Liothyrella neozelanica* is an endemic brachiopod that occurs throughout New Zealand with a latitudinal range that extends from 34°S to 54°S (Lee 1991) (Fig. 1.3). *L. neozelanica* occurs predominately at depths between 10 to 40 meters but has been found at depths down to 805 m (Foster 1974; pers. obs). *Liothyrella neozelanica* occurs either singly or in gregarious clumps, attached to either rock substrate or immobile animals that inhabit the substrate (Richardson 1981b).



**Figure 1.2** *Liothyrella uva* (Broderip 1833), Adelaide Island, Antarctica (Adapted from Lurman *et al.* 2010)





**Figure 1.3** A) *Liothyrella neozelanica* (Thomson 1918) brachiopod, Doubtful Sound, New Zealand. B). Rock wall community at 18 meters depth within Tricky Cove, Doubtful Sound (Photographs provided by Dick Singleton (A) and Mike Barker (B)).

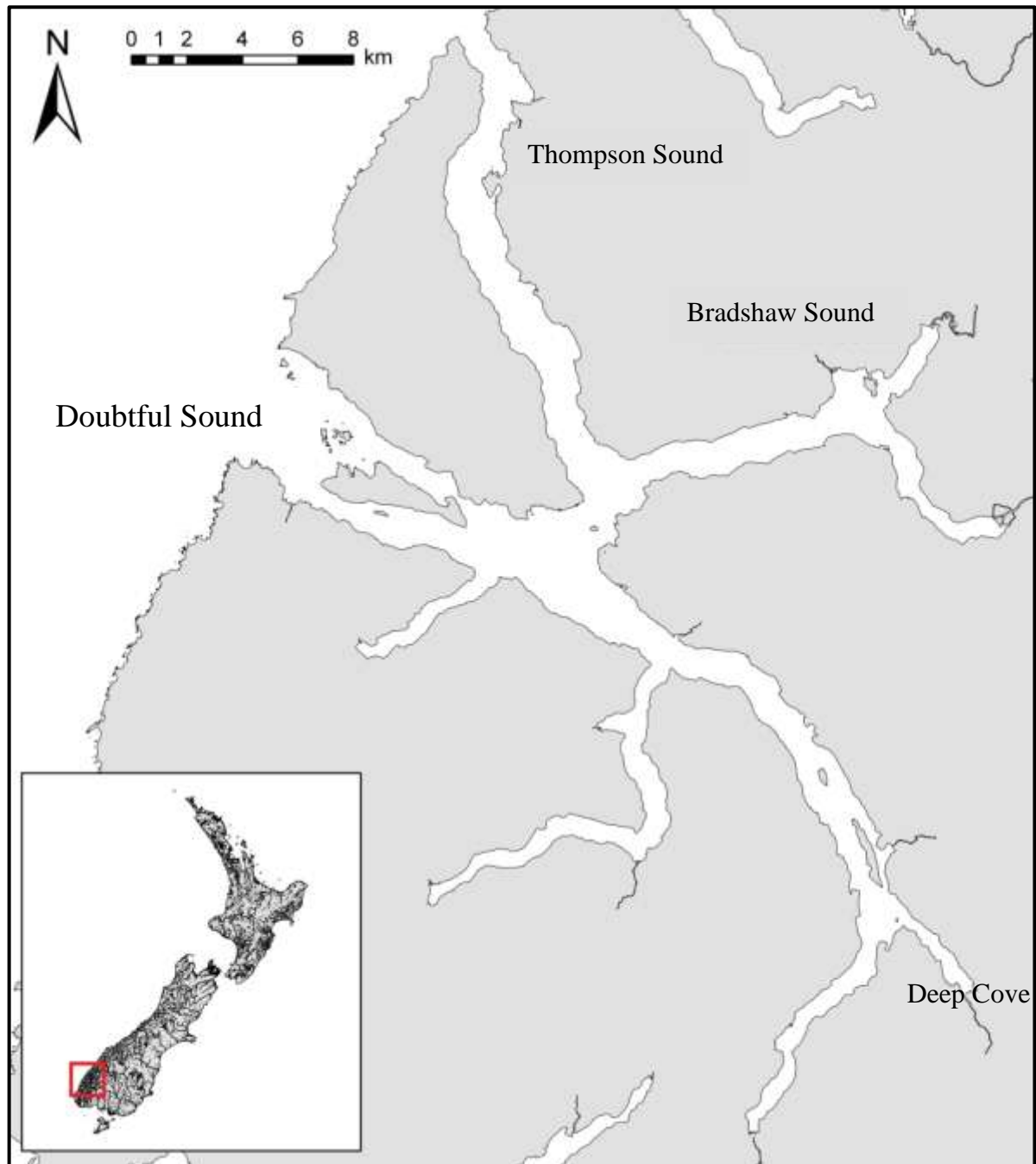


Previous research on *L. neozelanica* has been primarily focussed on reproduction, recruitment and ecology (Tortell 1981; Chuang 1994; Lee *et al.* 2010). Recently, physiological comparisons of *L. neozelanica* with *L. uva* have been determined in regards to differences in mitochondrial volume and densities (Lurman *et al.* 2010). The described research provides insight into the ecology, reproduction and physiology of *L. neozelanica*, however the overall biological knowledge of this species is limited and in most cases undefined. With the onset of climate change potentially affecting water temperatures, primary productivity and habitat ecology, it is important to understand the biology of New Zealand brachiopods to better understand how brachiopod communities within New Zealand will be affected. Furthermore, by gaining biological knowledge of the genus *Liothyrella*, direct comparisons between *L. uva* and *L. neozelanica* can be ascertained. This will provide greater insight into how species in the same genus have adapted to different environments and how they may be affected by climate change in the future.

### 1.3 Study site

The New Zealand brachiopod fauna consists of 26 genera and 50 species (MacFarlan *et al.* 2009; Lee *et al.* 2010). As a result, New Zealand is a recognised global hotspot for brachiopod diversity, representing 13% of all living brachiopod species (Dawson 1991; Emig 2009; Lee *et al.* 2010). Throughout New Zealand, brachiopods occur at relatively accessible depths (< 30 meters) in drowned valleys, harbours, the Hauraki Gulf, Stewart Island and in fiords such as Doubtful Sound (Rudwick 1962; Doherty 1979; Grange *et al.* 1981; Richardson 1981b). Due to their accessibility, diversity and often high abundances, New Zealand brachiopod anatomy, ecology and molecular phylogeny has figured prominently in international scientific literature (James *et al.* 1992; Peck 2001; Lee *et al.* 2010; Lurman *et al.* 2010). In particular, brachiopod communities within Doubtful Sound have become the focus of recent and current research (Ostrow *et al.* 2001; Lee *et al.* 2010; Lurman *et al.* 2010).

Doubtful Sound (45° 18'00" S, 166° 58' 45" E) is New Zealand's second largest fiord located on the west coast of the South Island in New Zealand (Fig. 1.4). Doubtful Sound stretches 40 km from the open ocean to the innermost cove and has a maximum depth of 465 meters (Lee *et al.* 2010). High levels of rainfall linked with steep sided fiord walls, results in a permanent low salinity layer (LSL) that ranges from 2-5 meters in depth (Smith and Witman 1999).



**Figure 1.4** Study site location, Doubtful Sound in the western South Island, New Zealand.

Epifaunal communities, made up primarily of mussels, barnacles, algae, bryozoans and hydroids dominate the first six meters of the fiord walls (Witman and Grange 1998). Below this level, light, sedimentation and primary productivity decrease, which in turn reduces bivalve prevalence (Rhodes and Thompson 1992; Lee *et al.* 2010). As a result, brachiopods flourish in depths between 10-30 meters making up ecologically important communities that are easily accessible for research (Grange and Singleton 1988; Witman and Grange 1998).

Five species make up the Doubtful Sound brachiopod community including *Liothyrella neozelanica*, *Notosaria nigricans*, *Terebratella sanguinea*, *Calloria inconspicua* and *Neoamula vector*. At some sites *Liothyrella neozelanica* occurs in high numbers, occupying large areas of the fiord walls between 10 to 30 meters depth. Due to the accessibility and prevalence of *Liothyrella*, Doubtful Sound was chosen as a suitable study site for research throughout 2011.

## 1.4 Climate Change

Climate change is now recognised throughout the scientific community with human activity projected to cause significant global warming during the 21<sup>st</sup> century (Kennedy *et al.* 2002). Although climate change is a natural phenomenon, human activity is substantially increasing its rate of change (Kennedy *et al.* 2002). Recent concern has focussed on how climate warming will affect life throughout the planet in the next century. Over the past 100 years the Earth has warmed with atmospheric temperatures increasing by 0.75°C (Jones *et al.* 1999). This gradual increase may appear insubstantial, but current models suggest that the Earth's near-surface temperature will increase between 1.1°C and 6.4°C and ocean temperatures will rise by around 2°C within the next century (IPCC 2007). Many marine species are susceptible to small temperature changes of just a few degrees (Kennedy *et al.* 2002), in particular those that are immobile and inhabit polar ecosystems (Peck 2008).

Climate change poses a significant threat to Antarctic marine invertebrates as many of them are immobile and have smaller thermal tolerances compared to their temperate counterparts, particularly in regards to raising metabolic rates to deal with increased temperatures (Peck 2008). Furthermore, the reproductive capabilities of many marine invertebrates are also predicted to be affected as thermal and photoperiod cues become disrupted. This may lead to localised extinctions (Lawrence and Soame 2004). Lastly, as water temperatures increase, some of the present day physiological barriers such as the sea temperature within Antarctic seas will be removed (Aronson *et al.* 2007). This will see immobile invertebrates face new predators, diseases, parasites and competition as mobile invertebrates and vertebrates migrate into their habitats (Stachowicz *et al.* 2002; Aronson *et al.* 2007; Cheung *et al.* 2009).

General brachiopod attributes including low metabolic rates, slow growth, immobilisation and requirements for hard substrates, makes them appear extremely susceptible to climate change in the future (Peck 2008). However, they have survived mass extinctions in the past and in terms of longevity are some of the most successful organisms extant (Richardson 1986). Due to their relative simplicity, wide latitudinal distribution, long history through mass extinctions and their potential susceptibility to climate change, *Liothyrella* brachiopods make ideal candidates to research the implications of climate change on marine invertebrates in the near future.

## 1.5 Research Objective

The overall objective of this research is to:

- **Determine the seasonal changes in biology of *L. neozelanica* so that direct comparisons with *L. uva* can be established to gain insight into how future environmental change may affect these two species.**

In order to achieve this objective the following specific aims will be addressed:

**Aim 1:** Determine the general biology of *Liothyrella neozelanica* by measuring the:

- Seasonal metabolism of *Liothyrella neozelanica*
- Seasonal biochemical composition in *Liothyrella neozelanica*
- Reproductive cycle of *Liothyrella neozelanica*
- Annual growth of *Liothyrella neozelanica*
- Environmental parameters present

**Aim 2:** Compare the seasonal biology of the temperate *L. neozelanica* to the Antarctic *L. uva* to gain insight into how warming sea temperatures resulting from climate change may affect brachiopods in the future.

By achieving the above aims, vital insight into how *Liothyrella* have adapted to a range of environments will be obtained. This will help the understanding of how regionally important species such as brachiopods have been able to adapt and survive through severe changes to their environments in the past. Furthermore, this information will provide clues into how *Liothyrella* will be affected by climate change in the future and may act as a building block, providing insight into how marine invertebrates will be affected as global warming continues.

## 1.6 Thesis outline

### Chapter 2: Seasonal metabolism of *Liothyrella neozelanica*

- The chapter introduces the study site and sampling methods before focusing on the seasonal metabolism of *Liothyrella neozelanica* throughout the 2011 study period. The seasonal changes in respiration rates are discussed and compared to the seasonal respiration rates of *Liothyrella uva*.

### Chapter 3: Seasonal biochemistry of *Liothyrella neozelanica*

- The chapter focuses on the seasonal biochemistry of *Liothyrella neozelanica* throughout the study period. Protein, lipid and carbohydrate levels are determined and compared to the seasonal biochemistry levels of *Liothyrella uva*.

### Chapter 4: Reproductive cycle of *Liothyrella neozelanica*

- The chapter determines the reproductive cycle of the endemic New Zealand brachiopod, *Liothyrella neozelanica*. A gonad index and histological results are used to discuss and determine the spawning period of *L. neozelanica*.

### Chapter 5: Annual growth of *Liothyrella neozelanica*

- The chapter concentrates on the annual growth rate of *Liothyrella neozelanica*, which is discussed in detail before being compared to the growth rate of the Antarctic *Liothyrella uva*.

At the end of the thesis, the biology of *L. neozelanica* is summarised and compared in detail to the biology of *L. uva* within the general discussion. Differences and similarities between the two species are established and linked to environmental factors. Insight into how each species has specifically adapted to each respective environment is discussed. Finally, the effects of climate change on the genus *Liothyrella* is discussed using biological indicators established throughout this study.



## CHAPTER 2

### SEASONAL METABOLISM OF *LIOTHYRELLA NEOZELANICA*



Respiration chambers holding *Liothyrella neozelanica* brachiopods within a water bath

**“If the animal did not habitually replace, through nourishing themselves, what they lose through respiration, the lamp would very soon run out of oil and the animal would perish, just as the lamp goes out when it lacks fuel ” (Antoine Lavoisier: 1743-1794)**



## 2.1 Introduction

Marine invertebrates are subject to various scales of seasonal and daily abiotic variation within their environment. Changes in temperature, photoperiod and food supply are some of the obstacles that marine invertebrates must tolerate and adapt to (Brockington and Clarke 2001). Temperature in particular, varies seasonally throughout the world's oceans with greatest variation occurring in temperate areas and least variation occurring at the poles and tropics (Peck *et al.* 2010). Temperature variation is often seen as a master regulator influencing growth rates, reproduction and the metabolic activity of marine invertebrates (Lurman *et al.* 2010). With climate change over the next century predicted to warm oceans by 2°C (IPCC 2007), it is important to understand the implications that increased sea temperature may have for the biology of marine invertebrates, in particular immobile invertebrates such as brachiopods.

Metabolism in the form of oxygen consumption has been shown to be positively correlated with temperature (Schmidt-Nielsen 1997). A 10°C increase in temperature typically causes the rate of oxygen consumption to increase by a factor of 2 to 3 within the majority of marine invertebrates (Schmidt-Nielsen 1997). This temperature effect is known as the  $Q_{10}$  and is defined by the following equation:

$$Q_{10} = \left( \frac{R_2}{R_1} \right)^{\left( \frac{10}{T_2 - T_1} \right)}$$

where  $R_1$  and  $R_2$  represent the respiration rate at temperature  $T_1$  and  $T_2$  respectively. It is generally accepted that an average  $Q_{10}$  of 2.5 reflects a normally behaving biological process and  $Q_{10}$  values greater than 5 indicate the influence of stress on a biological system (Clarke 1983).

Respiration rates in the form of oxygen consumption have been measured for several temperate brachiopods including three New Zealand species; *Terebratella sanguinea*, *Terebratella inconspicua* and *Neothyris lenticularis* (Shumway 1982; La Barbera 1986; Thayer 1986). It was found that the respiration rates of temperate brachiopods at 10°C were 3 to 7 fold lower than those rates observed in temperate molluscs of similar size (10 mg dry

weight (DW)) (Shumway 1982). The observed difference in respiration between temperate brachiopods and molluscs was attributed to the lophophore filaments within brachiopods not being fused together. This makes it difficult for brachiopods to create a high pressure difference between the shell and surrounding water, resulting in an inferior ability to pump water and ventilate compared to similarly sized molluscs (Rudwick 1970; Shumway 1982).

The respiration rates of Antarctic and temperate animals have been shown to differ, with temperate animals generally having higher respiration rates compared to their Antarctic counterparts (Rakusa-Suszczewski *et al.* 1976; Houlihan and Allan 1982; Peck *et al.* 1987; Davenport 1988). Such latitudinal differences in respiration rates have been shown to occur over a wide range of invertebrates including bivalves, gastropods, copepods and brachiopods. It has been found that the Antarctic bivalve *Yoldia eightsi* and the Antarctic gastropod *Nacella concinna* respire 2 to 4 times more slowly than the temperate bivalve *Mytilus edulis* and the temperate gastropod *Nucella lapillus* respectively (Houlihan and Allan 1982; Davenport 1988). Furthermore, Rakusa-Suszczewski *et al.* (1976) found that the Antarctic copepod *Rhincalanus gigas* respire 3 to 20 times slower than the temperate copepod *Euterpina acutifrons*. The respiration rate of the Antarctic brachiopod *L. uva* has been thoroughly investigated by Peck *et al.* (1986, 1987, 1997) who found that the respiration rate of *Liothyrella uva* at ambient temperature (0°C) was 12-times lower than those rates exhibited by temperate brachiopods at 10°C (Shumway 1982; Peck *et al.* 1986). The difference in respiration between Antarctic and temperate invertebrates is considered a reflection of the adaptations that Antarctic invertebrates have made to the low temperatures and extreme seasonality in primary productivity within the Southern Ocean (Peck *et al.* 1987; Peck 1996; Brockington and Clarke 2001; Brockington and Peck 2001; Kowalke *et al.* 2001).

Primary productivity, measured in chlorophyll concentration, has been shown to directly influence the respiration rates of Antarctic invertebrates through increased feeding (Brockington and Clarke 2001; Brockington and Peck 2001). It has been estimated by Brockington and Clarke (2001) that 80-85% of metabolic change in the Antarctic sea urchin *Sterechinus neumayeri* is attributed to increased feeding and growth rates associated with increased levels of chlorophyll concentration. Within temperate environments it is difficult to attribute changes in the respiration rates of marine invertebrates with singular environmental variables as photoperiod, temperature, salinity and chlorophyll concentrations often change

and coincide with one another throughout a single sampling period (Brockington and Clarke 2001). However, due to the relative stability and known seasonality of environmental factors within the Southern Ocean, it has been shown that temperature and chlorophyll concentration are the major environmental drivers influencing respiration rates in Antarctic marine invertebrates (Peck *et al.* 1987; Brockington and Clarke 2001; Brockington and Peck 2001; Peck and Veal 2001). Due to this it can be expected that the majority of metabolic changes in temperate marine invertebrates can also be attributed to fluctuations in temperature and chlorophyll concentration.

Previous comparisons of respiration rates between temperate New Zealand brachiopods and *L. uva* have all involved brachiopods from different genera being tested at different temperatures (0°C and 10°C). This has made it difficult to draw clear conclusions about the metabolic differences between Antarctic and New Zealand brachiopods (Peck *et al.* 1987). Furthermore, the effect of temperature on New Zealand brachiopod respiration has not been researched, with all previous testing being held at a constant 10°C (Shumway 1982). Comparisons of respiration rates between temperate brachiopods and *L. uva*, have shown *L. uva* to have a respiration rate that is 12 to 4 times lower than those of temperate brachiopods (Peck *et al.* 1986, 1987). This range is inconclusive with numerous variables including temperature and technique differing between studies (Shumway 1982; Peck *et al.* 1986, 1987). In order to obtain a more realistic comparison of metabolic ability between New Zealand and Antarctic brachiopods, it is necessary to determine the thermal limitations and capabilities of each. Moreover, greater clarity will be achieved if brachiopod species from each respective environment are more closely related.

The temperate brachiopod *Liothyrella neozelanica* is abundant within Doubtful Sound, New Zealand, where it can experience seasonal temperatures ranging from 8°C to 18°C (Lee 1991; Witman and Grange 1998). This broad temperature range differs substantially to that experienced by the Antarctic *L. uva*, with sea temperatures within the Southern Ocean typically changing by 2°C to 3°C (Brockington and Peck 2001; Peck *et al.* 2010). Due to the large temperature change ( $\approx 10^\circ\text{C}$ ) that *L. neozelanica* can experience annually, it is important to understand how its metabolism correspondingly adjusts. By ascertaining knowledge of *L. neozelanica*'s seasonal metabolic scope and the effect temperature has on it, a direct comparison with *L. uva* (Peck *et al.* 1986, 1987, 2010) can be established, providing insight

into how the genus *Liothyrella* has adapted metabolically over an evolutionary time scale to large changes in its ambient environments. Moreover, insight into how brachiopods have adapted their metabolism to inhabit a diverse range of habitats will be gained using *Liothyrella* as a base model.

### 2.1.1 Chapter objective

1. The primary objective of this chapter is to determine the seasonal metabolic scope of *Liothyrella neozelanica* within Doubtful Sound, New Zealand. In order to achieve this objective the following aims will be addressed:

- Determine the seasonal respiration response of *Liothyrella neozelanica* to annual temperature and chlorophyll concentration within Doubtful Sound
- Determine the thermal tolerance of *Liothyrella neozelanica*

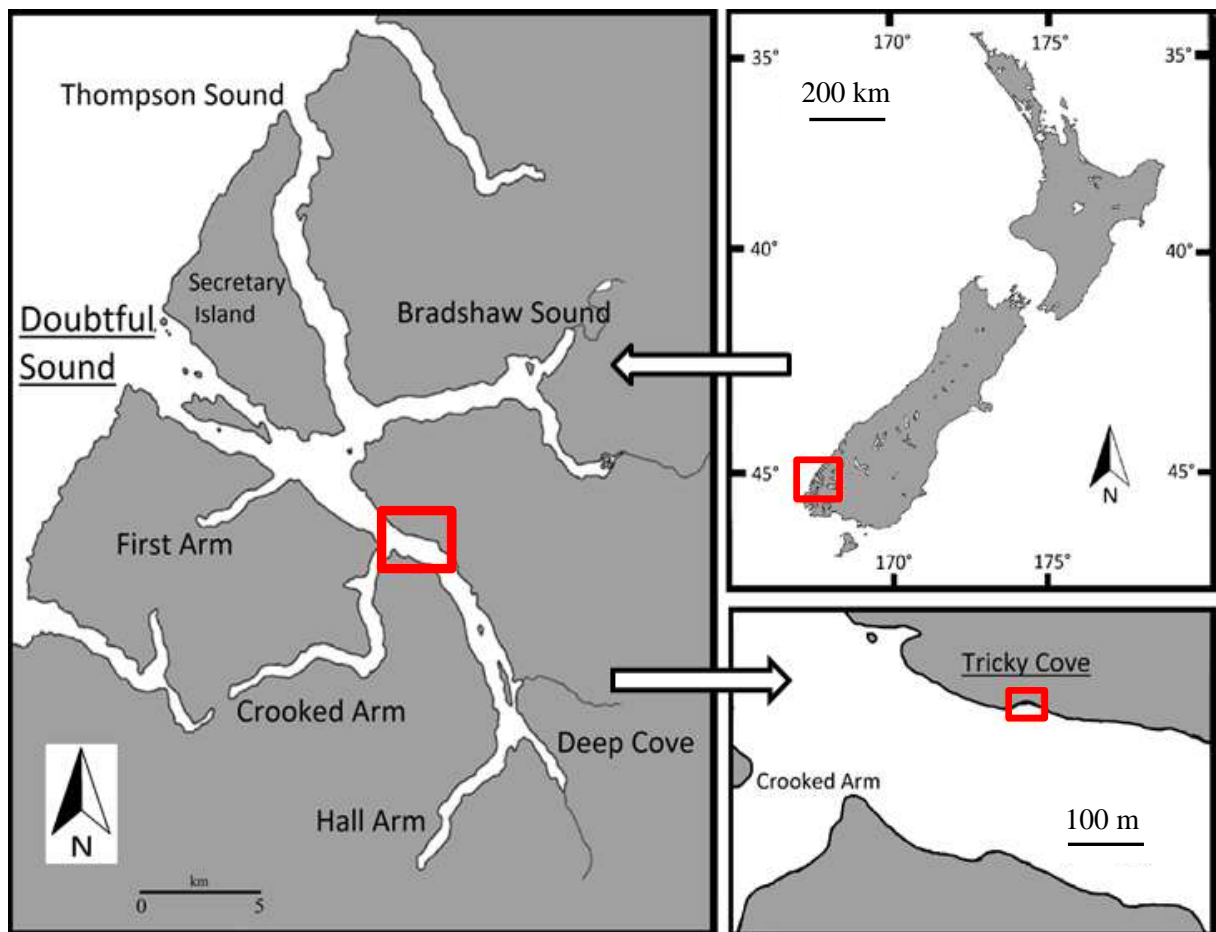
2. The secondary objective of this chapter is to compare the metabolic scopes of *Liothyrella neozelanica* and *Liothyrella uva*, to gain insight into how the genus *Liothyrella* may be affected by climate change (ocean warming) in the next century. The following aims will be addressed:

- Compare the seasonal metabolic rates and thermal tolerances of *Liothyrella neozelanica* and *Liothyrella uva*
- Gain insight into how the genus *Liothyrella* has specifically adapted to a wide range of environments
- Determine the effects increased sea temperature may have on the genus *Liothyrella* in the future

## 2.2 Methods

### 2.2.1 Study site and sampling

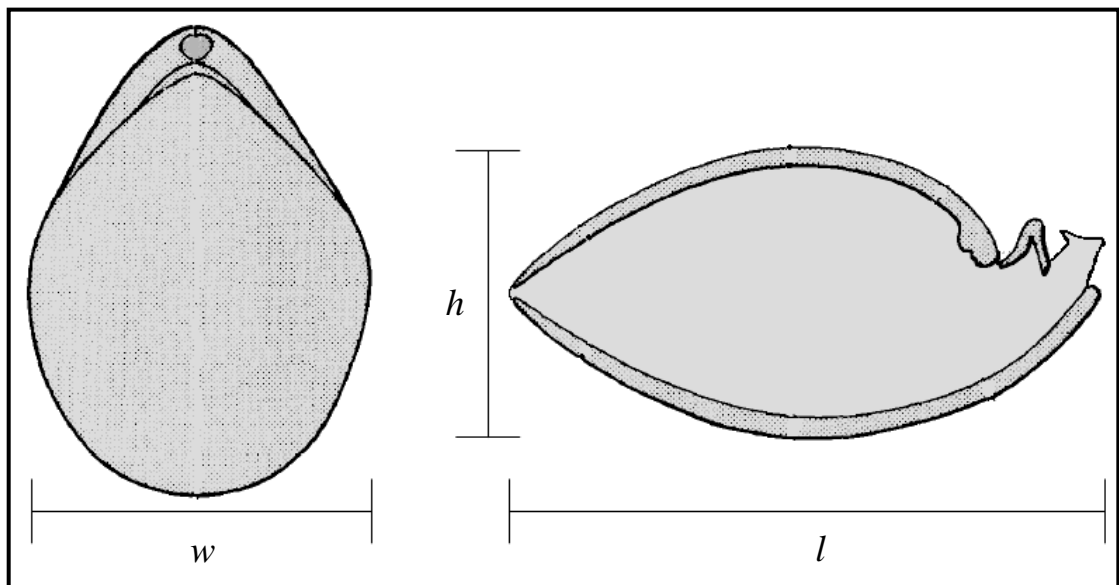
*Liothyrella neozelanica* brachiopods were collected on 10 separate occasions between December 2010 and December 2011 from the west wall of Tricky Cove in Doubtful Sound, New Zealand (Long: 45° 20' 47.84" S; Lat: 167° 02' 38.09" E) (Fig. 2.1). Each collection sampled sixty *L. neozelanica* brachiopods using SCUBA from depths ranging from 16 to 22 meters. Brachiopods were the dominant benthos between these depths with densities of 50 to 200 / m<sup>2</sup>. During sampling, *L. neozelanica* often died through shell fracture or by the loss of their pedicle when pulled from the wall by their shells. To avoid this each brachiopod was removed by pulling at the base of the pedicle to effectively detach it from the wall whilst causing minimal damage to the brachiopod itself. A total of 600 *L. neozelanica* brachiopods were collected over the study period (Dec 2010 – Dec 2011).



**Figure 2.1** Collection site of *L. neozelanica* at Tricky Cove within Doubtful Sound, New Zealand.

During each collection a standard Conductivity, Temperature, Depth (CTD) profile was taken using either a Seacat SBE 19-03 CTD or XR-620 RBR recorder, and a 10 litre water sample was collected from a depth of 18 meters. Water samples were immediately filtered at the University of Otago's Deep Cove field station within Doubtful Sound (Fig. 2.1) and the filter paper was transported on ice back to the Portobello Marine Laboratory (PML) in Dunedin, New Zealand for chlorophyll extraction. Chlorophyll levels were determined using a standard acetone extraction and were used together with information from the CTD profile to establish the environmental conditions present during each collection.

After each collection the brachiopods were transported back to PML and placed in a standard flow-through tank supplied with water from Otago Harbour. Before research was conducted on *L. neozelanica*, they were each individually tested for health following methods described by Rhodes and Thompson (1993). If individuals were visibly gaping in the tank, rapidly snapped shut if disturbed and resisted opening when pressure was lightly applied to the shell exterior, they were determined healthy and were used for research. The morphometric measurements of each brachiopod including the length (mm), width (mm) and height (mm) were measured using electronic vernier callipers to the nearest 0.01 of a millimetre so that morphometric relationships could be established (Fig. 2.2).

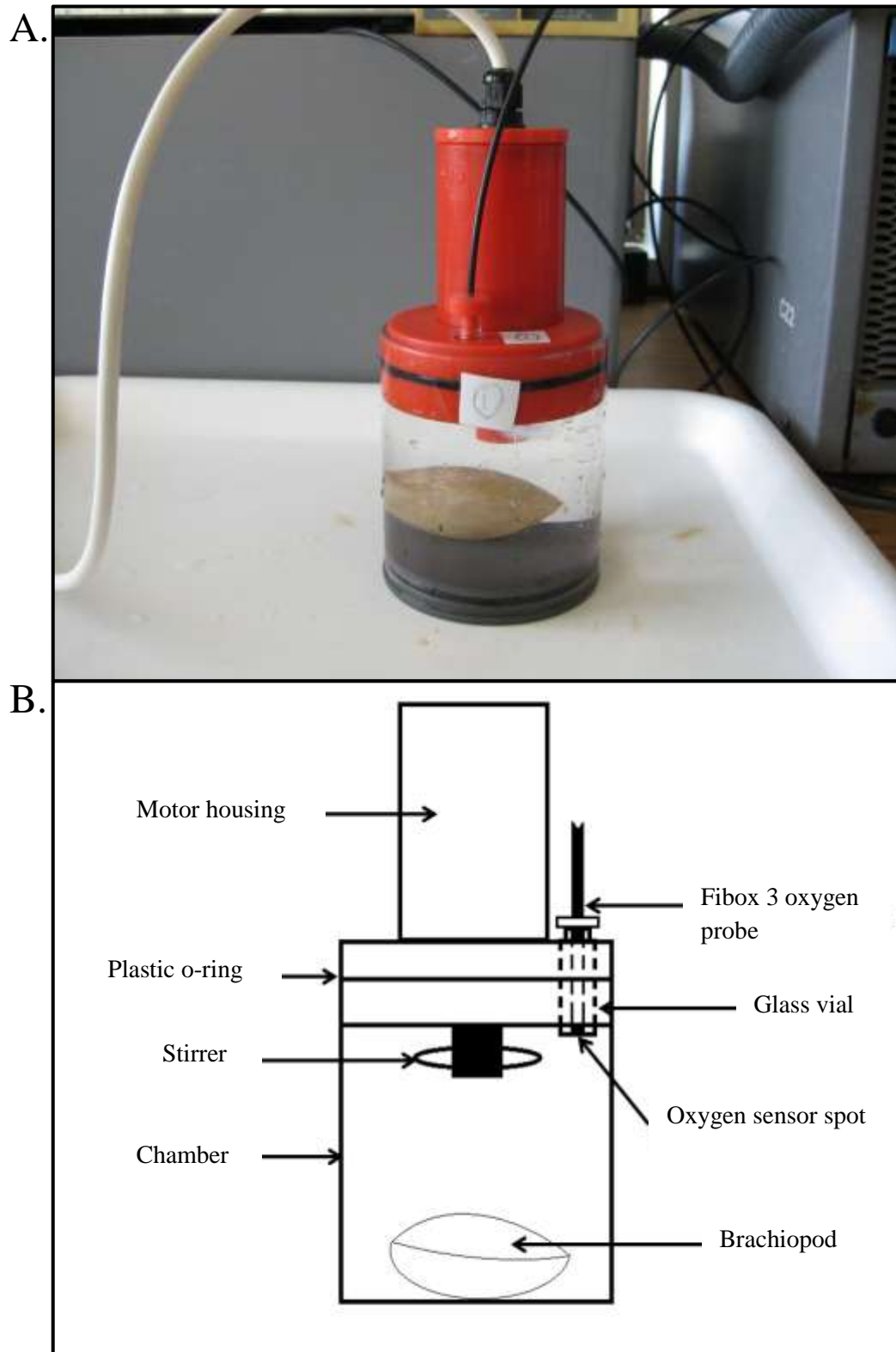


**Figure 2.2** Dimensions of *Liothyrella neozelanica* ( $h$  = height (mm),  $l$  = length (mm),  $w$  = width (mm) (Adapted from Peck and Holmes 1989a).

### 2.2.2 Respiration

After each collection (Ref. 2.2.1) eight *L. neozelanica* brachiopods were transported back to PML and placed into a holding tank within a CT room (Appendix 1.1). The CT room was set to the ambient temperature recorded at 18 meters depth in Tricky Cove at the time of collection (Appendix 1.2). The brachiopods were cleaned, paying close attention to remove all of the colonising organisms so that respiration rates would not be compromised during testing. Once cleaned, the brachiopods were kept at ambient temperature for a minimum of 48 hours to recover from handling and travel stress before being tested. The respiration rate of each individual *L. neozelanica* brachiopod was found across an array of temperatures including 8°C, 13°C, 18°C, 23°C and the ambient sea temperature at 18 meters depth during the time of collection (Appendix 1.3).

Following acclimation, each brachiopod was transferred from the CT room holding tank into a testing chamber that held acclimated 1 $\mu$ m filtered sea water (Fig. 2.3). To each chamber airtight lids with incorporated magnetic stirrers were fitted and oxygen bubbles were carefully removed. Each lid possessed an opening through which a Presens Pst3 oxygen sensor (attached to the bottom of a small glass vial) was pushed to become submerged within the chamber. O-rings around the lids and in the glass vial openings ensured the chambers became airtight once all of the components were fitted. Each chamber was placed into a water bath set to the required experimental temperature ( $\pm 0.1^\circ\text{C}$ ). A Fibox 3 oxygen probe was used to measure the initial oxygen concentration (mg/L) within the chambers. During testing care was taken when introducing the oxygen probe not to knock the chambers as this would cause the brachiopods to close, affecting respiration rates. Oxygen concentrations were measured every 30 minutes over a six hour period or until oxygen concentrations reached 75% of initial saturation levels. Oxygen concentrations obtained within the first hour of testing were methodically discarded as an initial lag period was observed where individuals remained closed due to handling during set up. Peck *et al.* (1987) found that the lag period for the Antarctic *L. uva* lasted for 3 hours during testing, however opening of *L. neozelanica* was often observed between 30 minutes to one hour. Therefore, a lag period of one hour was considered appropriate.



**Figure 2.3** (A) A respiration chamber with *Liothyrella neozelanica*. (B) Schematic representation of a respiration chamber.



The amount of oxygen used by each animal was calculated as the difference between the initial and final oxygen concentrations within the chambers. The water volume in each chamber was measured at the end of each experiment to allow for the volume occupied by the animal. The oxygen concentration ( $mg/L$ ) was corrected to allow for the volume of water and divided into an hourly rate to provide the amount of oxygen consumed per hour by each animal ( $\mu g(O_2) \text{ animal}^{-1} \text{ hr}^{-1}$ ). To express the respiration rates as the amount of oxygen consumed by organic tissue, the ash free dry mass (AFDM) of each brachiopod was determined at the end of testing. The AFDM was calculated as the difference between the dry weight (48hrs at  $60^\circ C$ ) and the ash weight found after ignition in a muffle furnace ( $470^\circ C$  for 12 hours). The overall respiration rate of each animal was defined as the amount of oxygen consumed per gram of organic tissue per hour ( $\mu g (O_2) g^{-1}(AFDM) \text{ hr}^{-1}$ ). A  $Q_{10}$  was calculated to see the effect a  $10^\circ C$  increase of temperature had on the respiration rates throughout the experimental period. The  $8^\circ C$  and  $18^\circ C$  respiration rates were used to calculate  $Q_{10}$  values as these temperatures approximated the temperature range naturally occurring in Tricky Cove ( $8\text{-}18^\circ C$ ) (Lee 1991).

### 2.2.3 Statistical analysis and comparisons

Statistical analyses were carried out using the JMP 7 statistical package. One-way ANOVA's were used to determine the differences in seasonal respiration rates among  $8^\circ C$ ,  $13^\circ C$ ,  $18^\circ C$ ,  $23^\circ C$  and ambient sea temperatures. Tukey–Kramer comparison tests were used to specify differences in respiration rates among months throughout the sampling period. In order to relate temperature and chlorophyll levels to the seasonal ambient respiration rates, bivariate regressions and a two-way ANOVA were used.

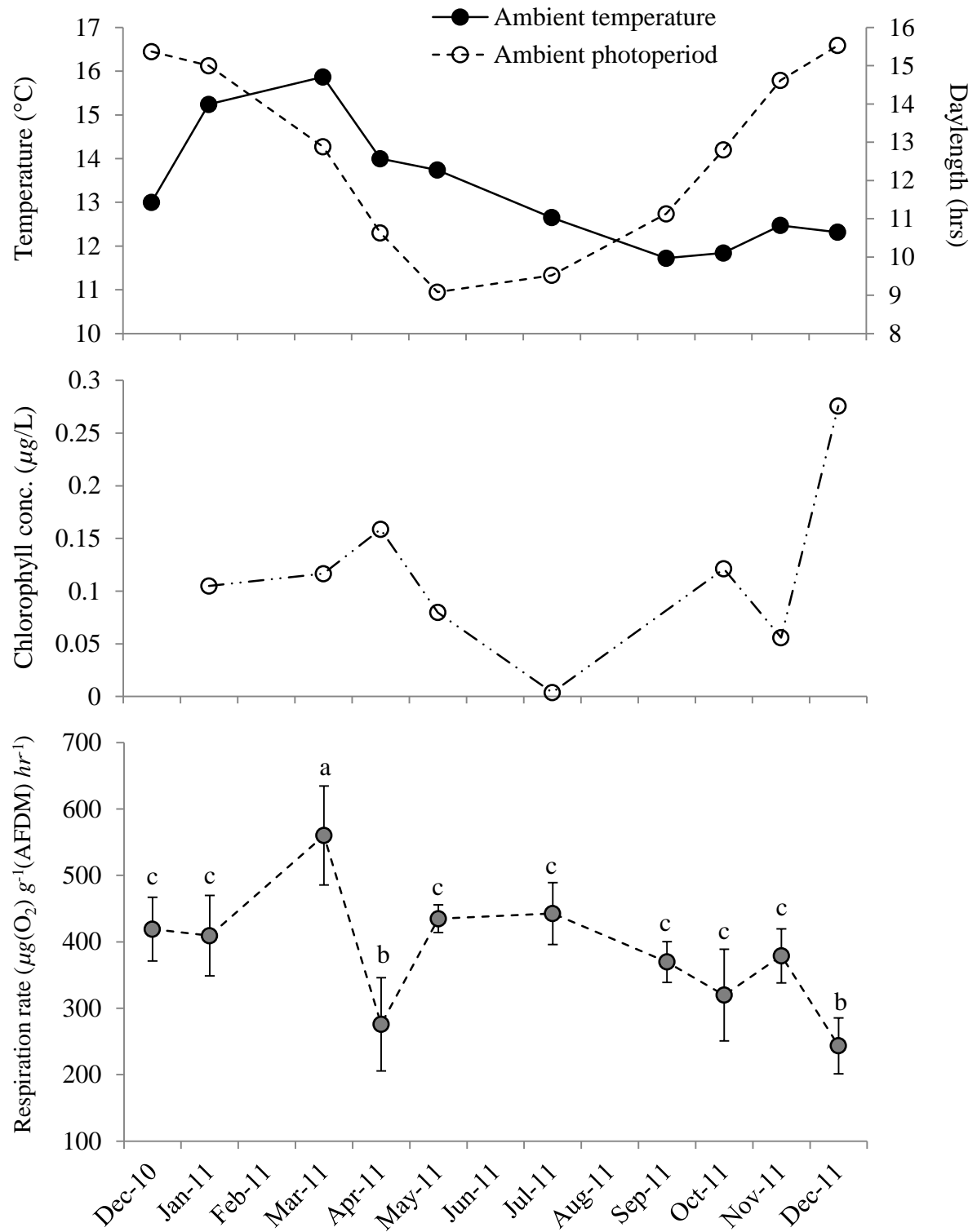
## 2.3 Results

### 2.3.1 Environmental results

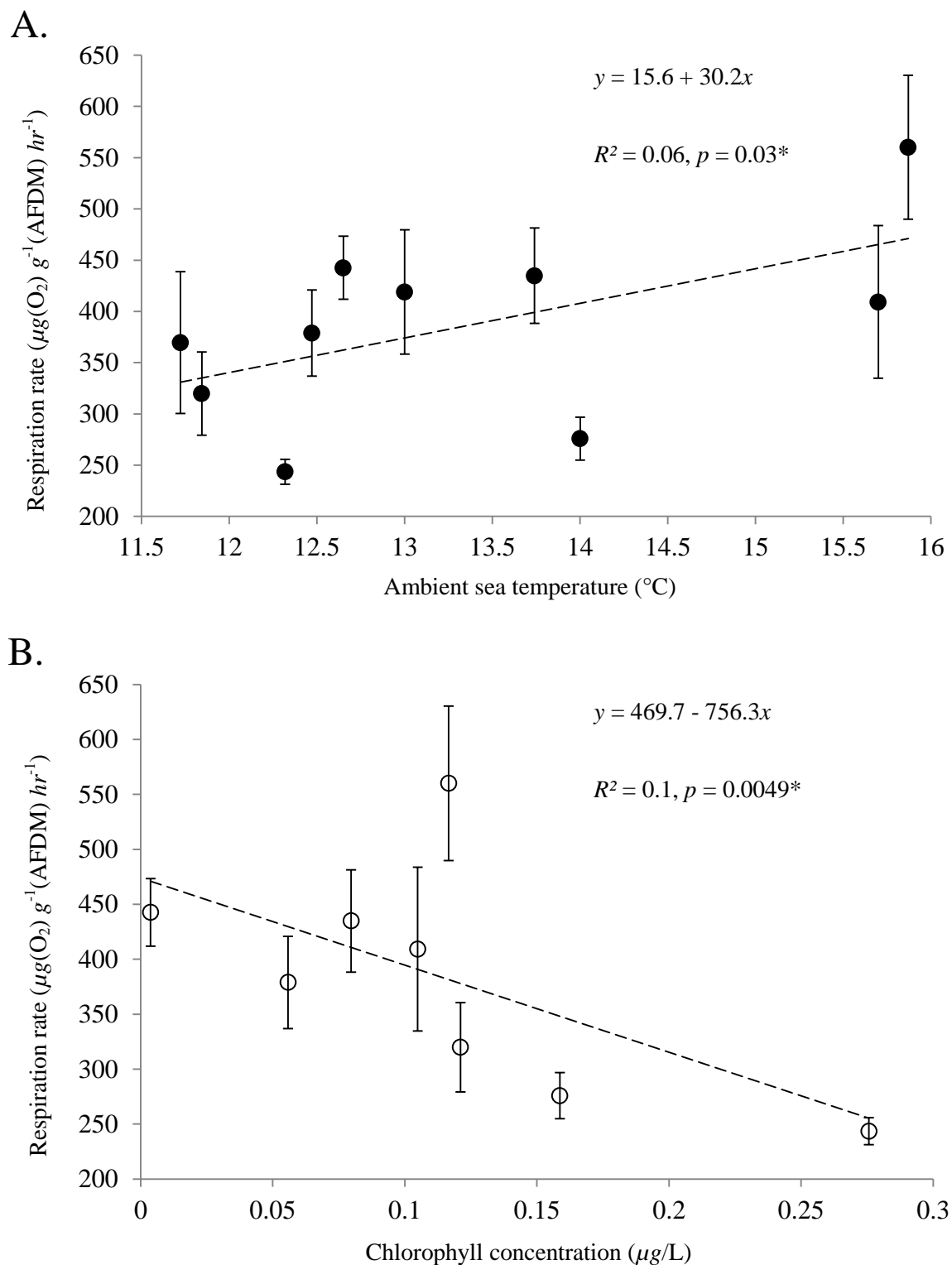
The ambient sea temperature at 18 meters depth within Tricky Cove ranged between 11.72°C and 15.8°C throughout the sampling period (December 2010 and December 2011) producing a 4.2°C seasonal change in temperature (Fig. 2.4). Chlorophyll levels fluctuated throughout the sampling period with lowest concentrations recorded in July 2011 (0.003  $\mu\text{g/L}$ ) and highest concentrations occurring in December 2011 during an apparent phytoplankton bloom (0.28  $\mu\text{g/L}$ ) (Fig. 2.4). The photoperiod within Tricky Cove ranged between 15.53 hours in the summer and 9.08 hours in the winter (Fig. 2.4). Salinity was relatively stable throughout the study, ranging between 33.8 and 34.93 psu.

### 2.3.2 Ambient respiration

Mean ambient respiration rates of *Liothyrella neozelanica* ranged from 243.5 to 560  $\mu\text{g}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$  between December 2010 and December 2011 (Fig. 2.4). Ambient respiration rates were significantly different throughout the sampled months ( $F = 2.8409$ ,  $df = 75$ ,  $p = 0.0069$ ). A Tukey-Kramer comparison test between months showed that the respiration rate in March 2011 (560  $\mu\text{g}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$ ) was significantly higher compared to the mean respiration rates of the remaining months (Fig. 2.4). The average ambient respiration rates observed in April 2011 (275.9  $\mu\text{g}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$ ) and December 2011 (243.5  $\mu\text{g}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$ ) were significantly lower compared to other months, but were not significantly different from each other ( $t = 1.33$ ,  $df = 14$ ,  $p = 0.2$ ) (Fig. 2.4). Ambient respiration rates generally rose and fell with increasing / decreasing ambient sea temperature (Fig. 2.4) and a linear regression between the two revealed a significant correlation ( $F = 4.9$ ,  $df = 75$ ,  $p = 0.03$ ) (Fig. 2.5A).

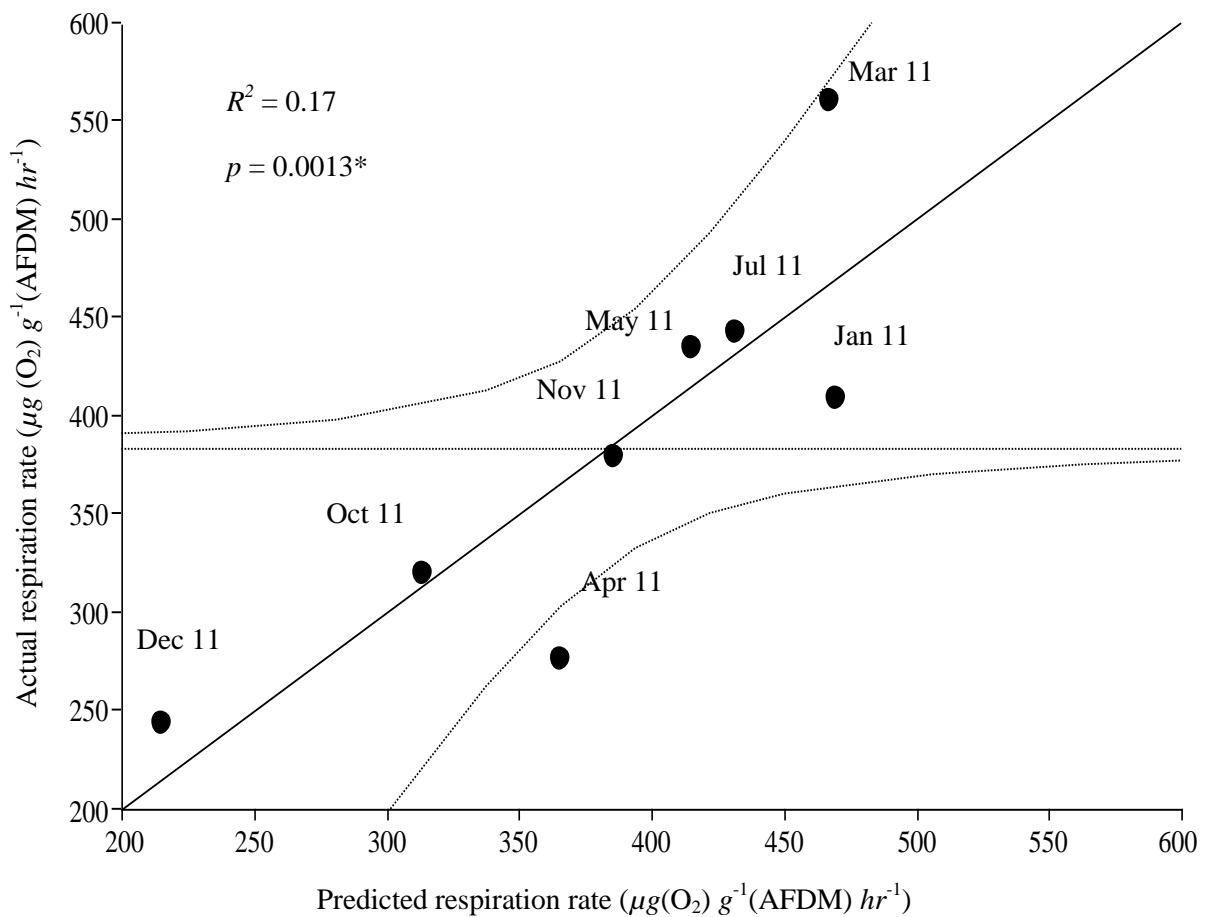


**Figure 2.4** Annual ambient sea temperature (°C), photoperiod (hours), chlorophyll concentration (µg/L) and ambient respiration rates (µg(O<sub>2</sub>) g<sup>-1</sup>(AFDM) hr<sup>-1</sup>) of *Liothyrella neozelanica* within Tricky Cove (± 1 s.e.) (*n* = 8 for each respiration rate, with exceptions in March 2011 (*n* = 6), September 2011 (*n* = 7) and November 2011 (*n* = 7)). Different lower case letters denote significant difference (*p* < 0.05).



**Figure 2.5** (A) Linear regression of monthly respiration rates vs. ambient sea temperature ( $n = 76$ ). (B) Linear regression of monthly respiration rates vs. ambient chlorophyll concentration ( $n = 61$ ) (\* denotes significance).

There was a trend of decreasing ambient respiration rate with increasing chlorophyll concentration (Fig. 2.4) and a linear regression between the two revealed a significant correlation ( $F = 8.42$ ,  $df = 75$ ,  $p = 0.0049$ ) (Fig. 2.5B). A two-way ANOVA revealed the combined effect of ambient sea temperature and chlorophyll concentration on the ambient respiration rate was significant ( $F = 7.33$ ,  $df = 75$ ,  $p = 0.0013$ ). Actual respiration rates and predicted respiration rates produced a significant correlation with chlorophyll concentration explaining 62% of the observed trend and temperature explaining the remaining 38% (Fig. 2.6).



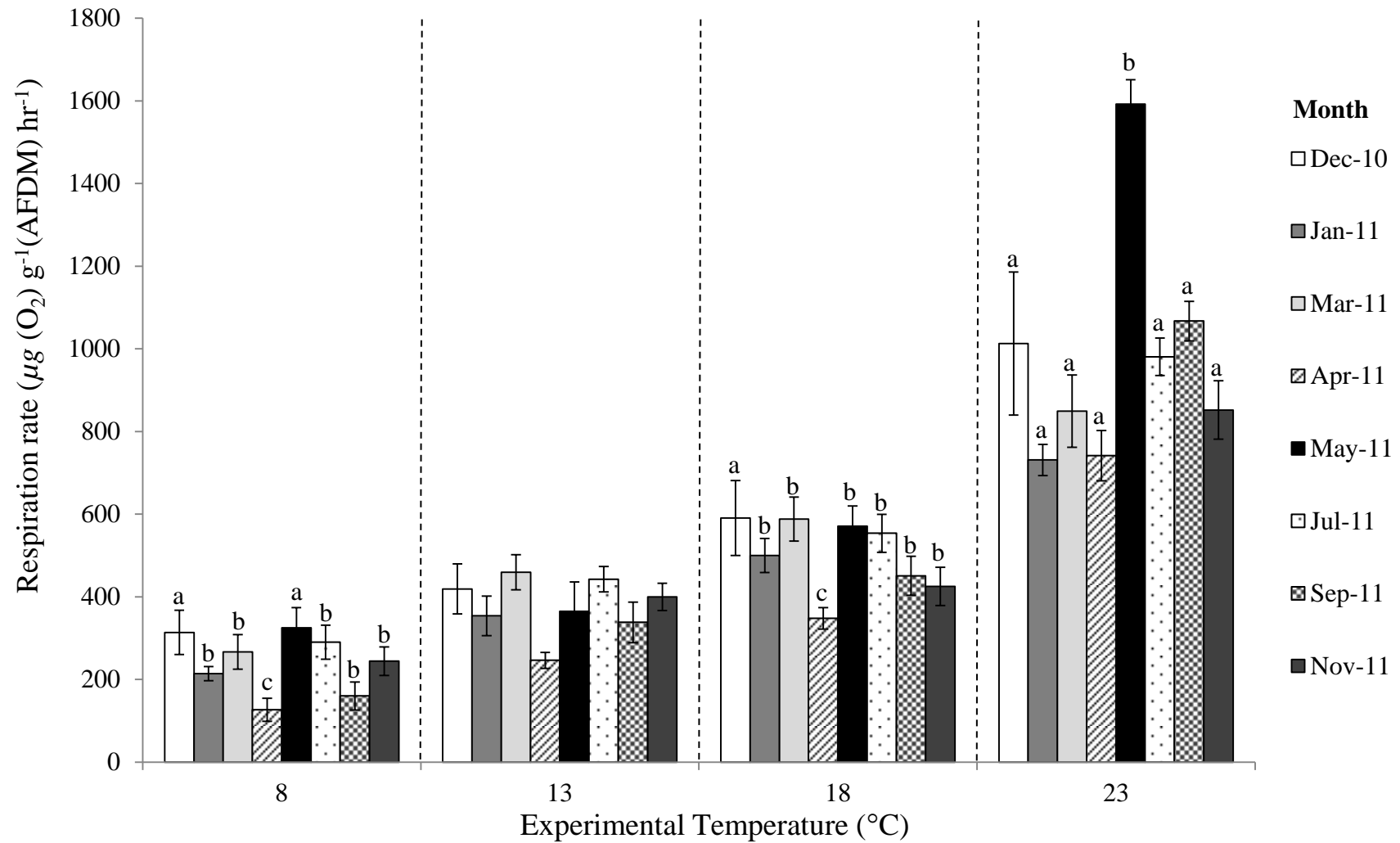
**Figure 2.6** Linear regression ( $y = 59.48 + 30.95 \cdot \text{Temp} - 767.1 \cdot \text{Chlorophyll}$ ) of actual *L. neozelanica* ambient respiration rates and predicted respiration rates from a two-way ANOVA, examining the combined effects of sea temperature and chlorophyll concentration (95% C. I.; horizontal dotted line represents average respiration rate;  $n = 8$  / respiration rate; \* denotes significance).

### 2.3.3 Effect of experimental temperature on respiration

For each sampled month, the average respiration rate increased across the range of temperature treatments (8°C, 13°C, 18°C, 23°C), with  $Q_{10}$  values ranging between 1.74 (November 2011) and 2.81 (September 2011) (Fig. 2.7, Table 2.1). A two-way ANOVA determined respiration rates across temperature treatments were significantly different from one another ( $F = 11.06$ ,  $df = 31$ ,  $p < 0.0001$ ), and respiration rates in May 2011 were significantly different from the remaining sampled months. Average monthly respiration rates at 8°C ( $F = 3.46$ ,  $df = 59$ ,  $p = 0.0041$ ), 18°C ( $F = 2.59$ ,  $df = 59$ ,  $p = 0.023$ ) and 23°C ( $F = 6.21$ ,  $df = 50$ ,  $p < 0.0001$ ) were significantly different throughout the sampling period, while average respiration rates at 13°C were not significantly different from one another ( $F = 2.05$ ,  $df = 59$ ,  $p = 0.07$ ) (Fig. 2.7). Tukey - Kramer comparison tests revealed the average respiration rates in April 2011 at 8°C and 18°C were significantly lower compared to the respiration rates in other months at each respective temperature (Fig. 2.7).

**Table 2.1** Average *Liothyrella neozelanica* respiration rates across the sampling period at 8°C and 18°C with corresponding  $Q_{10}$  values (Average  $Q_{10}$  value across the sampling period = 2.2).

Month	8°C Oxygen consumption ( $\mu\text{g}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$ )	18°C Oxygen consumption ( $\mu\text{g}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$ )	$Q_{10}$
December 2010	197.78	590.71	1.88
January 2011	313.92	500.30	2.33
March 2011	214.28	588.19	2.20
April 2011	266.86	348.03	2.74
May 2011	126.95	571.25	1.76
July 2011	325.12	553.64	1.91
September 2011	290.18	450.74	2.81
November 2011	160.23	425.08	1.74



**Figure 2.7** Average respiration rates from each sampling month at 8°C, 13°C, 18°C and 23°C. (Lower case letters denote significant difference ( $p < 0.05$ ) between months for each individual temperature treatment. Nb. no significant differences observed between months at 13°C).

The average respiration rate in December 2010 at 18°C ( $591 \mu\text{g}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$ ) was significantly higher than the remaining sampled respiration rates at 18°C. Furthermore, the average respiration rates at 8°C in December 2010 ( $314 \mu\text{g}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$ ) and May 2011 ( $325 \mu\text{g}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$ ) were significantly higher compared to the other sampled respiration rates at 8°C (Fig. 2.7). At higher temperatures (18°C and 23°C) respiration rates appeared relatively stable throughout the sampled months with the exceptions of April 2011 at 18°C ( $127 \mu\text{g}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$ ) and May 2011 at 23°C ( $1592 \mu\text{g}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$ ), which were significantly lower and higher respectively (Fig. 2.7). When the monthly oxygen consumption rates at each temperature (8°C, 13°C, 18°C, 23°C) were plotted against the ambient temperature of each month (Appendix 1.4), the ambient sea temperature had no significant effect ( $p > 0.05$ ) on the change in respiration of *L. neozelanica* in response to temperature treatments.



## 2.4 Discussion

### 2.4.1 Annual change in ambient respiration and sea temperature

Annual ambient respiration rates of *Liothyrella neozelanica* changed significantly throughout the sampling period, often responding to changes in ambient sea temperature. Temperature is regarded as a master regulator of marine ectotherms, influencing reproduction, growth and metabolic processes (Brockington and Clarke 2001; Hochachka and Somero 2002; Lurman *et al.* 2010). A positive correlation between respiration and temperature has been shown to occur over a wide range of marine invertebrates including bivalves, copepods and echinoderms (Widdows 1973; Rakusa-Suszczewski *et al.* 1976; Brockington and Clarke 2001). Although the effects of temperature on marine invertebrate respiration is well known (Ikeda 1977; Stickle and Bayne 1982; Ivleva 1980; Luxmoore 1984; Spanopoulos-Hernández *et al.* 2005), there is limited information concerning the effects of temperature on brachiopod respiration, with the majority of previous research being focussed on the Antarctic brachiopod *L. uva* (Peck *et al.* 1986, 1987, 2010). Moreover, the effect of temperature on temperate brachiopod respiration is relatively undefined with previous research either involving one experimental temperature (10°C) (Shumway 1982) or two experimental temperatures (5.8°C and 10.7°C) (Peck *et al.* 1989). To date no information has been available about the annual response of respiration to changes in ambient sea temperature in New Zealand brachiopods or brachiopods in general.

Within this study, the ambient respiration rate of *L. neozelanica* ranged from 243.5 to 560  $\mu\text{g}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$  throughout the research period, showing *L. neozelanica* increases and decreases its respiration rate in response to surrounding environmental conditions. The ambient respiration rate of *L. neozelanica* often matched the trends shown by ambient sea temperature, with raised respiration (560  $\mu\text{g}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$ ) occurring in March 2011 when ambient sea temperature was warmest (15.8°C) and an apparent drop in respiration in April 2011 (276  $\mu\text{g}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$ ) when ambient sea temperature dropped (14°C). However, the relationship between ambient respiration and ambient temperature was not linear throughout the sampling period, as respiration rates often remained between 350 and 450  $\mu\text{g}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$  despite changes in ambient sea temperature of 2°C. A linear regression between ambient respiration and sea temperature produced a significant relationship, showing temperature causes some of the variation in ambient respiration seen

throughout the sampling period. However the fit of the linear regression was weak ( $R^2 = 0.06$ ) showing significant variation within the model. This variation can be explained by the majority of ambient respiration rates being significantly similar ( $p > 0.05$ ) throughout the sampling period despite the changes in ambient sea temperature. The exceptions were March 2011, April 2011 and December 2011.

The apparent stability in ambient respiration throughout the majority of sampled months, suggests *L. neozelanica* may be thermally compensating to the surrounding changes in ambient sea temperature throughout the year. Thermal compensation has been defined as the maintenance of an appropriate physiological rate in the face of temperature change (Clarke 1991). Metabolic compensation has been described in temperate marine invertebrates, including the periwinkle *Littorina littorea* and the bivalve *Mytilus edulis* (Newell and Pye 1970). The two species were able to maintain stable metabolic rates throughout an annual period, despite substantial change ( $10^{\circ}\text{C}$ ) to the surrounding ambient temperature (Newell and Pye 1970). It was suggested that enzyme-substrate affinities increase and the activation of different isozymes occurs in these species to keep oxygen consumption independent from temperature change (Newell and Pye 1970). More recent research involving summer and winter mitochondria in *L. neozelanica* showed that mitochondrial volume and cristae density increase in winter to combat the effects of colder temperatures on contractile rates observed in the adductor muscle (Lurman *et al.* 2010). The findings from these two studies together with the findings in this study suggest *L. neozelanica* regulates its respiration rates at a subcellular level in order to maintain a stable metabolic rate, despite sea temperature change throughout an annual period.

#### **2.4.2 Effect of experimental temperature on respiration**

The respiration rates of *L. neozelanica* consistently increased with increasing experimental temperature ( $8^{\circ}\text{C}$ ,  $13^{\circ}\text{C}$ ,  $18^{\circ}\text{C}$ ,  $23^{\circ}\text{C}$ ) throughout the sampling period. Temperature affects metabolic processes in two main ways: 1) As temperature increases, molecular processes obtain sufficient kinetic energy to reach required activation levels; 2) increasing temperature causes the equilibrium constant for any given reaction to shift leading to higher reaction rates (Hochachka 1991). As a consequence, chemical reactions speed up and the rate of oxygen consumption increases as energy (ATP) is consumed (Hochachka 1991). The respiration rates

of marine invertebrates generally increase by a factor of 2 to 3 with a 10°C increase in temperature ( $Q_{10}$ ) until a critical temperature is reached ( $T_c$ ), whereby aerobic metabolism transitions into anaerobic metabolism (Clarke 1983; Schmidt-Nielsen 1997; Pörtner *et al.* 1999). The  $Q_{10}$  effect on aerobic respiration has been shown to occur across a wide range of marine invertebrates, including the Antarctic brachiopod *Liothyrella uva* which exhibits  $Q_{10}$  values ranging from 1.36 to 3.09 (Widdows 1973; Peck 1989; Schmidt-Nielsen 1997; Allan *et al.* 2006; Peck *et al.* 2010).

Within this study, the  $Q_{10}$  values of *L. neozelanica* ranged between 1.74 and 2.81 with an average  $Q_{10}$  rate of 2.2 occurring throughout the sampling period. This average  $Q_{10}$  value lies between the generally accepted range of 2 to 3 shown in other marine invertebrates (Clarke 1983). Interestingly, apart from the months of December 2010, April 2011 and May 2011, the respiration rates of *L. neozelanica* responded to temperature treatments with no significant difference between months throughout the sampling period. This finding suggests that *L. neozelanica* has a consistent broad thermal tolerance range, enabling it to cope with the wide-ranging annual ambient sea temperatures (8 - 18°C) that can be experienced within Doubtful Sound (Lee 1991; Witman and Grange 1998).

The apparent broad thermal range exhibited by *L. neozelanica* also agrees with Lee (1991) who noted that *L. neozelanica* must have a broad temperature tolerance based on its wide latitudinal distribution throughout New Zealand waters (34°S to 54°S), which can experience temperatures ranging from 5°C to >21°C. Moreover, *L. neozelanica* occurs at depths ranging from 10 to 805 meters (Foster 1974), again signifying its ability to withstand large temperature differences. Finally the response of *L. neozelanica* to the temperature treatments within this study, showed aerobic respiration still occurring at 23°C, signifying the critical temperature ( $T_c$ ) (where aerobic respiration switches to anaerobic respiration) had not been reached (Pörtner *et al.* 1999; Peck *et al.* 2002). Wide latitudinal distribution, together with a large depth distribution and the ability to aerobically respire between 8°C and 23°C, shows that *L. neozelanica* possesses a large metabolic tolerance to temperature.

Interestingly, the highest respiration rates of *L. neozelanica* ( $1592 \mu\text{g}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$ ) were recorded for 23°C in May 2011. This respiration rate was found to be significantly

different from the respiration rates exhibited at 23°C in other sampled months. When the May 2011 individuals were dissected after testing at 23°C, the lophophore in four of the eight individuals had either gone limp, lost all structural form, or in some cases appeared to have been broken down completely. It is possible that May 2011 *L. neozelanica* individuals were unable to tolerate the testing temperature of 23°C and had undergone autolysis. Interestingly, the same May 2011 individuals had significantly higher respiration rates at 8°C compared to all of the remaining months apart from December 2010. The higher respiration rates at lower temperatures, coupled with less tolerance to higher temperature (23°C), initially indicated that *L. neozelanica* may have been acclimating to colder temperatures throughout the winter. However, this was not consistent with observations from other months which showed no further evidence of acclimation towards colder temperatures throughout the sampling period, despite further cooling of the ambient sea temperature. Therefore the observed high respiration rates recorded in May 2011 are likely to be a result of experimental error.

At 13°C, there were no significant differences in respiration rates between the sampled months, despite the ambient sea temperature fluctuating above and below this temperature throughout the sampling period. Due to the lack of variation in response to 13°C throughout the sampling period and due to 13°C being the mid-point of the temperature range experienced in Doubtful Sound (8 – 18°C), it seems reasonable to suggest that the metabolism of *L. neozelanica* functions optimally at or close to 13°C.

### **2.4.3 Ambient chlorophyll effect on respiration**

Ambient chlorophyll concentration fluctuated throughout the sampling period with highest rates occurring in December 2011 (0.28 µg/L) and lowest rates occurring in July 2011 (0.004 µg/L), which is seasonally consistent with previous findings for Doubtful Sound (Goebel *et al.* 2005). Within temperate environments it is often difficult to attribute the effects of chlorophyll to respiration rates as they often co-vary with temperature. As a result, the majority of research determining chlorophyll effects on respiration has involved Antarctic species, due to the relative stability in temperature found within the Southern Ocean. It has been shown that increasing chlorophyll concentrations positively influence the respiration rates of Antarctic marine invertebrates via increased feeding rates (Peck 1996; Peck 1998; Peck and Veal 2001; Brockington and Clarke 2001; Brockington and Peck 2001).

Brockington and Clarke (2001) determined that a rise in respiration rate over summer exhibited by the Antarctic sea urchin *Sterechinus neumayeri*, was mainly attributed (85%) to increased levels of chlorophyll concentration as opposed to temperature (15%). Moreover, Peck (1996) found that increased feeding rates by *Liothyrella uva* caused a 1.6 – fold increase in basal respiration rates. Although these findings are associated with Antarctic marine invertebrates it is reasonable to assume that chlorophyll concentration has a similar effect on temperate marine invertebrate respiration.

Within this study, ambient respiration rates were found to be negatively affected by increased chlorophyll levels, with lowest ambient respiration rates occurring in April 2011 ( $276 \mu\text{g}(\text{O}_2) \text{g}^{-1}(\text{AFDM}) \text{hr}^{-1}$ ) and December 2011 ( $244 \mu\text{g}(\text{O}_2) \text{g}^{-1}(\text{AFDM}) \text{hr}^{-1}$ ), during raised chlorophyll concentrations ( $> 0.15 \mu\text{g/L}$ ). The apparent negative effect on the ambient respiration rate of *L. neozelanica* is contrary to previous studies that show increasing chlorophyll concentration positively influences respiration rates via increased feeding (Peck 1996; Peck 1998; Peck and Veal 2001; Brockington and Clarke 2001). The negative correlation between ambient chlorophyll concentration and the ambient respiration rate of *L. neozelanica* within this study, may be attributed to travel and handling stress affecting true respiration rates, however this is unlikely as all of the tested brachiopods were acclimated for 48 hours at the ambient Tricky Cove sea temperature to accommodate this. A possible explanation for the observed negative effect of chlorophyll concentration on *L. neozelanica* respiration is the nature of environmental sampling that took place within Tricky Cove.

Phytoplankton blooms within Doubtful Sound are often patchy in space and time, capable of rapidly appearing and dispersing over a matter of days (Goebel *et al.* 2005). The sampling that took place within this study represented a snapshot of the environmental variables occurring in Tricky Cove at one time during each sampled month. As a result, the chlorophyll samples may have been a misrepresentation of the true chlorophyll concentrations that *L. neozelanica* was subject to throughout each sampled month. Therefore, the negative correlation between *L. neozelanica* ambient respiration rate and ambient chlorophyll concentration found within this study should be viewed with caution until verified by future research. In order to more accurately determine the effects of environmental conditions on brachiopod ambient respiration in future research, weekly environmental samples or environmental loggers should be utilised to provide more detailed descriptions of environmental factors over the sampling

period. Furthermore, controlled feeding experiments involving varying levels of phytoplankton could provide further insight into the observed negative correlation between ambient chlorophyll concentration and ambient *L. neozelanica* respiration observed within this study.

#### 2.4.4 Combined temperature and chlorophyll effect

The combined effect of ambient temperature and chlorophyll concentration on the ambient respiration rate of *L. neozelanica* produced a model with greater significance and a better fit ( $R^2 = 0.17$ ) compared to the individual linear regressions involving separate factors. From the model, it was found that chlorophyll concentration explained 62% of the ambient respiration variation throughout the sampling period, while ambient temperature explained 32%. These findings suggest that ambient chlorophyll concentration, as opposed to ambient temperature, is the main environmental variable influencing the ambient respiration rate of *L. neozelanica* throughout an annual period. Brockington and Clarke (2001) showed that chlorophyll concentration was the main environmental driver (85%) of seasonal change in the ambient respiration of the Antarctic *S. neumayeri* through increased feeding and growth rates associated with phytoplankton blooms. This finding was confirmed with further seasonal respiration and excretion research on the echinoderm *S. neumayeri*, conducted by Brockington and Peck (2001).

Although the fore-mentioned findings within this study are consistent with those found for *S. neumayeri*, it should be noted that the ambient temperature range in Doubtful Sound (10°C) is far greater than that found within the Southern Ocean ( $\pm 3^\circ\text{C}$ ). This difference in seasonal temperature range makes it more likely that ambient temperature has a larger effect on the seasonal change of *L. neozelanica* ambient respiration compared to that of *S. neumayeri*. This is confirmed by ambient temperature explaining 32% of the seasonal change in ambient respiration in *L. neozelanica* compared to 15% in *S. neumayeri* (Brockington and Clarke 2001). Importantly, although the results for *L. neozelanica* are consistent with previous findings, it should again be noted that the effect of chlorophyll concentration on the ambient respiration rate of *L. neozelanica* within this study should be viewed with caution (as mentioned in section 2.4.3) and may cause less of an effect on the ambient respiration of *L. neozelanica* than exhibited by the model.

### 2.4.5 *Liothyrella* comparisons

Respiration rates of *Liothyrella uva* under simulated summer and winter conditions were investigated by Peck *et al.* (1986, 1987) who found that the summer respiration rate of *L. uva* is 13% higher than winter respiration rates as a result of temperature and increased feeding during the highly productive summer months within the Southern Ocean. Both the summer and winter respiration rates of *L. uva* were directly compared by Peck *et al.* (1986, 1987) to the respiration rates of several temperate brachiopod species based on previous investigations by Shumway (1982) and La Barbera (1986). It was concluded by Peck *et al.* (1987) that the respiration rates of *L. uva* were 8.8 to 12.9 times lower than temperate brachiopod respiration rates. However, the techniques used between studies differed substantially with respiration rates from Shumway's investigations being based on internal tissue dry weight only, while Peck's respiration rates were based on the overall AFDW of entire individuals. Due to these discrepancies in methodologies between studies, it is difficult to draw precise conclusions on the differences between respiration rates of temperate and Antarctic brachiopods.

Previous research by Peck *et al.* (1986, 1987) measured the oxygen consumption of *Liothyrella uva* in  $\mu\text{l}$  of oxygen as opposed to  $\mu\text{g}$  of oxygen. In order to directly compare the respiration rates of *L. neozelanica* and *L. uva*, the ambient oxygen consumption rate of *L. neozelanica* in  $\mu\text{g}$ , was converted into the amount of oxygen in  $\mu\text{g}$ -atoms by dividing by 16. The amount of oxygen consumed in  $\mu\text{g}$  – atoms was then further converted into  $\mu\text{l}$  of oxygen by multiplying by 11.2 (Peck and Uglow 1990). The transformed seasonal ambient respiration rates of *L. neozelanica* are directly compared to the simulated summer and winter respiration rates of *L. uva* in Table 2.2. The summer respiration rate of *L. neozelanica* was found to be 5.6 times higher than that shown by *L. uva* under simulated summer conditions, while the winter respiration rate of *L. neozelanica* was 7.8 times higher than that of *L. uva* under simulated winter conditions. It should be noted however, that the testing temperatures differ substantially between the seasons and species involved in these comparisons (Table 2.2). To account for temperature differences between this and previous *L. uva* studies, an average  $Q_{10}$  temperature correction of 2.2 (based on the average  $Q_{10}$  of *L. neozelanica*) was applied to a 1 gram (AFDW) summer and winter *L. neozelanica* individual to predict the respiration rates of *L. neozelanica* at 0°C (Table 2.3).

**Table 2.2** Oxygen consumption rate ( $\mu\text{l}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$ ) comparisons between the Antarctic *Liothyrella uva* (Peck *et al.* 1987) and the temperate *Liothyrella neozelanica* brachiopods (Temperature is calculated as an average based on the months within each season).

Species	Season	Temp ( $^{\circ}\text{C}$ )	<i>n</i>	Oxygen consumption ( $\mu\text{l}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$ )
<i>Liothyrella uva</i>	Simulated summer	0	45	46
<i>Liothyrella uva</i>	Simulated winter	0	105	38
<i>Liothyrella neozelanica</i>	Summer (Dec-Feb)	13.5	23	258
<i>Liothyrella neozelanica</i>	Autumn (Mar-May)	14.5	22	288
<i>Liothyrella neozelanica</i>	Winter (June-Aug)	12.7	8	298
<i>Liothyrella neozelanica</i>	Spring (Sep-Nov)	12	22	246

**Table 2.3** Oxygen consumption rate ( $\mu\text{l}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$ ) comparisons between the Antarctic *Liothyrella uva* (Peck *et al.* 1987) and the temperate *Liothyrella neozelanica* brachiopods at  $0^{\circ}\text{C}$ . (\* indicates theoretical values that have been calculated based on an average  $Q_{10}$  of 2.2 from original values shown in Table 2.2).

Species	Season	Temp ( $^{\circ}\text{C}$ )	Oxygen consumption ( $\mu\text{l}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$ )
<i>Liothyrella uva</i>	Simulated summer	0	46
<i>Liothyrella uva</i>	Simulated winter	0	38
<i>Liothyrella neozelanica</i>	Summer	0	89*
<i>Liothyrella neozelanica</i>	Winter	0	109.5*



The  $Q_{10}$  temperature correction produced *L. neozelanica* summer and winter respiration rates of  $89 \mu\text{l}(\text{O}_2) \text{ g}^{-1}(\text{AFDW}) \text{ hr}^{-1}$  and  $109.5 \mu\text{l}(\text{O}_2) \text{ g}^{-1}(\text{AFDW}) \text{ hr}^{-1}$  respectively at  $0^\circ\text{C}$ . These corrected respiration values are 1.9 times and 2.9 times higher than *L. uva* in the summer and winter respectively. These comparisons suggest *L. uva* has to some degree cold-adapted its metabolism by lowering its ambient respiration rate to account for the extreme seasonality in primary production and low temperature range found within the Southern Ocean (Peck *et al.* 1986; Peck 1996). These findings agree with previous comparisons between Antarctic and temperate brachiopods (Peck *et al.* 1986, 1987). However the results in this study used more closely related methodologies and brachiopods from the same genus, providing a more robust comparison.

#### 2.4.6 Climate change implications

Climate change in the future is expected to raise mean sea temperature around the world by  $2^\circ\text{C}$  (IPCC 2007). By increasing the ambient sea temperature in Doubtful Sound ( $8 - 18^\circ\text{C}$ ) by  $2^\circ\text{C}$ , the maximum sea temperature that could occur is  $20^\circ\text{C}$  (Lee 1991; Witman and Grange 1998). This is well within the aerobic scope of *L. neozelanica* found within this study as aerobic respiration was still occurring at  $23^\circ\text{C}$ , showing the critical temperature ( $T_c$ ) had not yet been met. However, it can be expected that a  $2^\circ\text{C}$  rise in temperature will increase respiration rates by 19% from current levels exhibited at  $18^\circ\text{C}$  ( $y = 15.6 + 30.2x$ , Fig. 2.5), suggesting that energy will be consumed at higher rates. Consequently, food availability and energy utilisation will most likely play a key role in the ability of *L. neozelanica* to adapt to rising sea temperature in the next century.

In contrast to *L. neozelanica*, Peck *et al.* (2010) found that *L. uva* showed no signs of acclimation to temperatures only  $1^\circ\text{C}$  to  $2^\circ\text{C}$  above the maximum ambient sea temperature within the Southern Ocean, making *L. uva* potentially more vulnerable to climate change over the next century.

### 2.4.7 Conclusion

The research conducted in this chapter has shown that *Liiothyrella neozelanica* respiration is influenced by a combination of parameters within its environment, namely chlorophyll concentration and temperature which have negative and positive effects respectively on the ambient respiration of *L. neozelanica*. The respiration rate of *L. neozelanica* increased uniformly across a range of temperature treatments with an average  $Q_{10}$  value of 2.2 falling between the generally accepted range of 2 to 3. *L. neozelanica* showed evidence of thermal compensation throughout the sampling period, suggesting it actively adjusts its metabolism in accordance with surrounding environmental conditions. The ability of *L. neozelanica* to respire aerobically between the temperatures of 8°C and 23°C suggests *L. neozelanica* has a broad thermal tolerance range.

On comparison with *L. uva*, the summer and winter respiration rates of *L. neozelanica* are 1.9 times and 2.9 times higher, even after a  $Q_{10}$  temperature correction is applied. This suggests *L. uva* has to some degree cold-adapted its metabolism in response to the low temperature range and extreme seasonal productivity within the Southern Ocean. From the findings within this study it is expected that *L. neozelanica* will be able to cope with and adapt to climate change in the next century, whereas *L. uva* may be particularly vulnerable, due to its inability to acclimate to higher ocean temperatures predicted to occur within its environment over the next century.



# CHAPTER 3

## SEASONAL BIOCHEMISTRY OF *LIOTHYRELLA NEOZELANICA*



Dynex Opsys Microplate reader with loaded protein samples

**“Organic chemistry is the chemistry of carbon compounds. Biochemistry is the study of carbon compounds that crawl” (Mike Adams)**

### 3.1 Introduction

Extensive research has been conducted on the seasonal biochemistry of benthic marine invertebrates in order to distinguish nutrition, seasonal energetics, feeding strategies and the importance of biochemistry in reproduction, development and physiology (Ansell 1974, 1975; Taylor and Venn 1979; Davis and Wilson 1983; Beninger and Lucas 1984; Gallagher *et al.* 1998; Hill *et al.* 2008). Typically, research has focussed on the major biochemical constituents; protein, lipid and carbohydrate. Protein is regarded as the major biochemical constituent within the majority of animals, determining tissue structure, being used as a metabolic energy source and functioning in numerous biological pathways as enzymes (Robbins 1983). Lipid serves several purposes, most commonly as an energy storage product due to its high energy content per unit of weight compared to protein and carbohydrate (Hill *et al.* 2008). Carbohydrate is important in providing structure and form and is used as a chemical specific energy source for certain tissues and metabolic processes (Hill *et al.* 2008). Typically carbohydrate concentrations are far less than that of lipid and protein due to its inferiority as an energy source (Hill *et al.* 2008).

Historically the majority of marine invertebrate biochemical research has focussed on molluscs, in particular bivalves, due to their important commercial value and prevalence (Ansell 1972, 1974; Bayne 1976; Davis and Wilson 1983). More recent attention has focussed on the seasonal biochemical composition of polar marine invertebrates, to understand their seasonal energetics in response to the extreme seasonality in primary productivity within these regions (Peck *et al.* 1987; Gallagher *et al.* 1998; Brockington 2001; Ahn *et al.* 2003). This research has shown that polar marine invertebrates generally use lipid in gametogenic processes, use carbohydrate in fuelling metabolism and rely heavily on protein to fuel metabolic processes; particularly over winter when food availability is low (Peck *et al.* 1987; Brockington 2001; Ahn *et al.* 2003). Temperate marine invertebrates however, exhibit a range of strategies to meet energy requirements. The temperate oyster *Ostrea edulis* and the banded wedge shell *Donax vittatus*, utilise carbohydrate and lipid as their main energy sources (Walne 1970; Ansell and Sivadas 1973), whereas temperate mussels, including *Mytilus edulis*, rely on protein and carbohydrate as their major energy sources (Bayne 1976; Zandee *et al.* 1980; Gabbott 1983). The differences between temperate biochemical comparisons together with differences between polar and temperate biochemical comparisons, highlight the

importance of understanding the seasonal biochemistry of individual marine invertebrate species.

Despite extensive biochemical research involving temperate and Antarctic marine invertebrates, the seasonal biochemistry of brachiopods is largely unknown, with only two published studies describing the seasonal biochemical composition of the Antarctic brachiopod *Liothyrella uva* (Peck *et al.* 1986, 1987). Furthermore as a consequence of this dearth of information, the seasonal biochemistry of *L. uva* has in the past been compared to the seasonal biochemistry of the temperate bivalve *Mytilus edulis* (Peck *et al.* 1986, 1987). These comparisons between *L. uva* and *M. edulis* have provided insight into how temperate and polar marine invertebrates differ; however, these species are from different phyla and temperate marine invertebrates show variation across species in how they seasonally utilise protein, lipid and carbohydrate. In order to fully quantify the seasonal biochemical adaptations made by an Antarctic species such as *L. uva* to its environment, it is necessary to compare its seasonal biochemistry with research involving a temperate brachiopod that is preferably closely-related.

The temperate New Zealand brachiopod *Liothyrella neozelanica* makes up ecologically important habitat within Doubtful Sound, colonising steep-sided walls throughout the fiord (Lee *et al.* 2010; pers. obs.). Despite its prevalence and relative importance to Doubtful Sound invertebrate communities, relatively little is known about its overall biology, in particular, how it adjusts its seasonal biochemistry to environmental change. Moreover, relatively little is known about the biology of New Zealand brachiopods in general, despite New Zealand being regarded as a “brachiopod global hotspot” (Lee *et al.* 2010). In order to understand the unique diversity and prevalence of brachiopods within New Zealand, it is vital to understand how they interact with the surrounding environment. By researching the seasonal biochemical composition of *L. neozelanica*, insight into how a temperate New Zealand brachiopod utilises and stores energy in response to environmental change will be achieved. Furthermore, this research will enable more appropriate temperate biochemical comparisons with *L. uva*, providing a more realistic understanding of how brachiopods have adapted to their respective environments.

### 3.1.1 Chapter objective

1. The primary objective of this chapter is to establish the seasonal changes in protein, lipid and carbohydrate within *L. neozelanica* over one year. This will be achieved by addressing the following aims:

- Determine total seasonal levels of protein, lipid and carbohydrate within *L. neozelanica*
- Determine the seasonal levels of protein, lipid and carbohydrate within each major internal component (lophophore, stomach, gonad) of *L. neozelanica*
- Determine the influence of ambient temperature and chlorophyll concentration on the biochemical concentrations of *L. neozelanica*

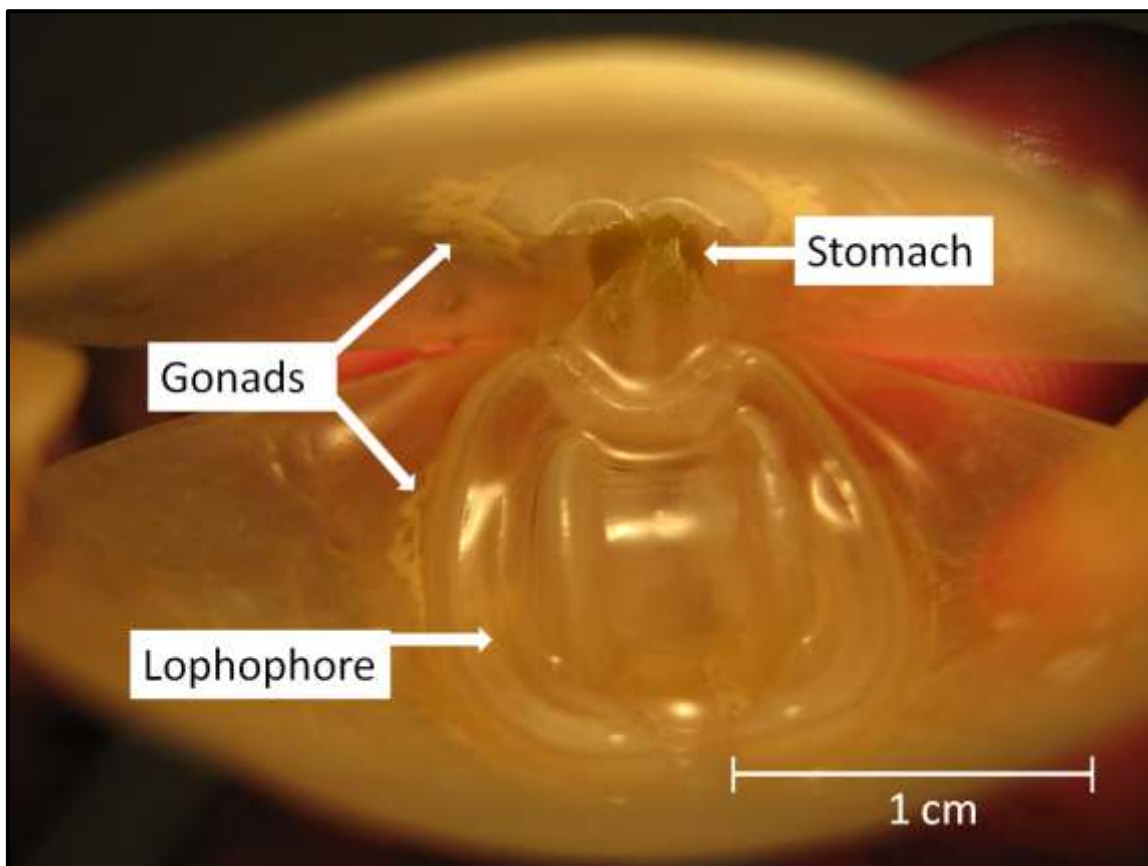
By achieving these aims the importance of each biochemical constituent within *L. neozelanica* will be determined, providing an understanding of how *L. neozelanica* seasonally adjusts its biochemistry to environmental change over an annual period.

2. The secondary objective of this chapter is to compare the seasonal biochemistry of *Liothyrella neozelanica* and *Liothyrella uva* to determine the biochemical adaptations each species has made to their respective environment.

## 3.2 Methods

### 3.2.1 Biochemical composition

From December 2010 to December 2011, thirty *Liothyrella neozelanica* individuals were collected every six weeks from Tricky Cove, Doubtful Sound and the ambient environmental conditions were recorded (Refer 2.2.1). All specimens of *L. neozelanica* were immediately dissected at Deep Cove into three major components; the lophophore, stomach (including the digestive gland) and gonads (Fig. 3.1). All other tissues were ignored and left in the shell. The components were immediately wrapped in tinfoil, stored in liquid nitrogen and transported to PML in Dunedin, where they were stored at -80°C. Of the thirty component replicates, protein, lipid and carbohydrate levels were determined using ten lophophores, ten stomachs and ten sets of gonads each. The components for each test were lyophilised for 48 hours using a Labconco 4.5 freeze drier so that the dry weight (DW) in mg could be determined. Each component was ground into fine particulates using a standard mortar and pestle before being tested.



**Figure 3.1** Major organs of a *Liothyrella neozelanica* brachiopod.



### 3.2.2 Soluble protein analysis

To quantify soluble protein concentration, ground samples of each component (lophophore, stomach, gonad) ranging from 1-2 mg (DW) were placed into separate test tubes containing 1ml distilled water. Each test consisted of thirty samples sourced from ten lophophores, ten stomachs and ten sets of gonads. Each sample was homogenised for 20 minutes in a DigiTech sonicator. From each sample, 25  $\mu$ l was taken and loaded into a 96-well microplate. A nine point, linear standard curve was created using pure bovine albumin with concentrations ranging from 0 to 2000  $\mu$ g/ml, and loaded into the microplate. To each sample and standard, 200  $\mu$ l of working reagent from a Bio-Rad Protein Assay kit (Bradford protein assay) was added. The plate was incubated at 37°C for 30 minutes to develop colour. A Dynex Opsys microplate reader was used to measure the absorbance of each sample at 562 nm. The absorbance of each sample was converted into the amount of soluble protein present ( $\mu$ g/ml) based on the bovine albumin standard curve. For further information refer to Appendix 2.1 and Appendix 5.

### 3.2.3 Lipid analysis

To quantify soluble lipid, ten 1 – 2 mg (DW) samples of each component were prepared following previously described methods (Ref. 3.2.1) and placed into test tubes. To each sample 2 ml of methanol:chloroform (1:1) solution was added and then sonicated for 20 minutes. A modified Van Handel analysis based on the methods described by Inouye and Lotufo (2006) was used to determine the levels of lipid within each sample using a sulfuric acid / vanillin reagent reaction (Appendix 2.2). A Dynex Opsys microplate reader (490 nm) was used to measure the amount of lipid ( $\mu$ g/ml) within each sample based on a nine point, pure soybean extract (100%) standard curve (0 - 2000  $\mu$ g/ml) (Appendix 2.2; Appendix 5).

### 3.2.4 Carbohydrate analysis

To quantify soluble carbohydrate concentration, ten 1-2 mg (DW) samples of each component were prepared following previously described methods (Ref. 3.2.1). To each sample 1ml of distilled water was added together with 250  $\mu$ l of tri-chloroacetic acid (T.C.A) and sonicated for 20 minutes. The phenol-sulphuric acid method described in Mann and Gallagher (1985), was used to determine the carbohydrate concentration within each sample (Appendix 2.3). A

Dynex Opsys microplate reader (490nm) was used to measure the amount of carbohydrate ( $\mu\text{g/ml}$ ) within each sample based on a nine point, pure glucose (100%) standard curve (0 - 500  $\mu\text{g/ml}$ ) (Appendix 2.3; Appendix 5).

### 3.2.5 Total biochemical weights

Throughout the sampling period, 98 *L. neozelanica* individuals were cleaned and dissected into four main components; the lophophore, gonads, stomach and shell. The amount of organic tissue present within each component as a function of ash-free dry mass (AFDM), was determined as the difference between the dry weight (48 hours at 60°C) and the weight after ignition in a muffle furnace (12 hours at 470°C). The AFDM weights were averaged to define the amount of organic tissue within each component of an average (160 mg AFDM) *L. neozelanica* individual. It was important to define the amount of organic tissue in this way to stay consistent with methods described in previous studies involving *Liothyrella uva* (Peck *et al.* 1987), to enable direct comparisons. Using these averages, the amount of each biochemical (mg) within the lophophore, stomach and gonads from each collection was calculated using proportions found from the tested samples (Ref. 3.2.2 - 3.3.4). These biochemical values within each component were then combined to produce an overall amount of protein, lipid and carbohydrate occurring in the main tissues of an average *L. neozelanica* individual throughout the sampling period.

### 3.2.6 Statistical analysis

All statistical analyses were carried out using the JMP 7 statistical package. One-way ANOVA's were used to distinguish monthly differences in total protein, lipid and carbohydrate. One-way ANOVA's were also used to determine biochemical differences within the lophophore, stomach and gonad of *L. neozelanica* throughout the sampling period. Tukey-Kramer tests were used to specify differences in biochemistry between the sampled months. Linear regressions were used to establish statistical relationships between biochemical constituents, ambient sea temperature and ambient chlorophyll concentration.

### 3.3 Results

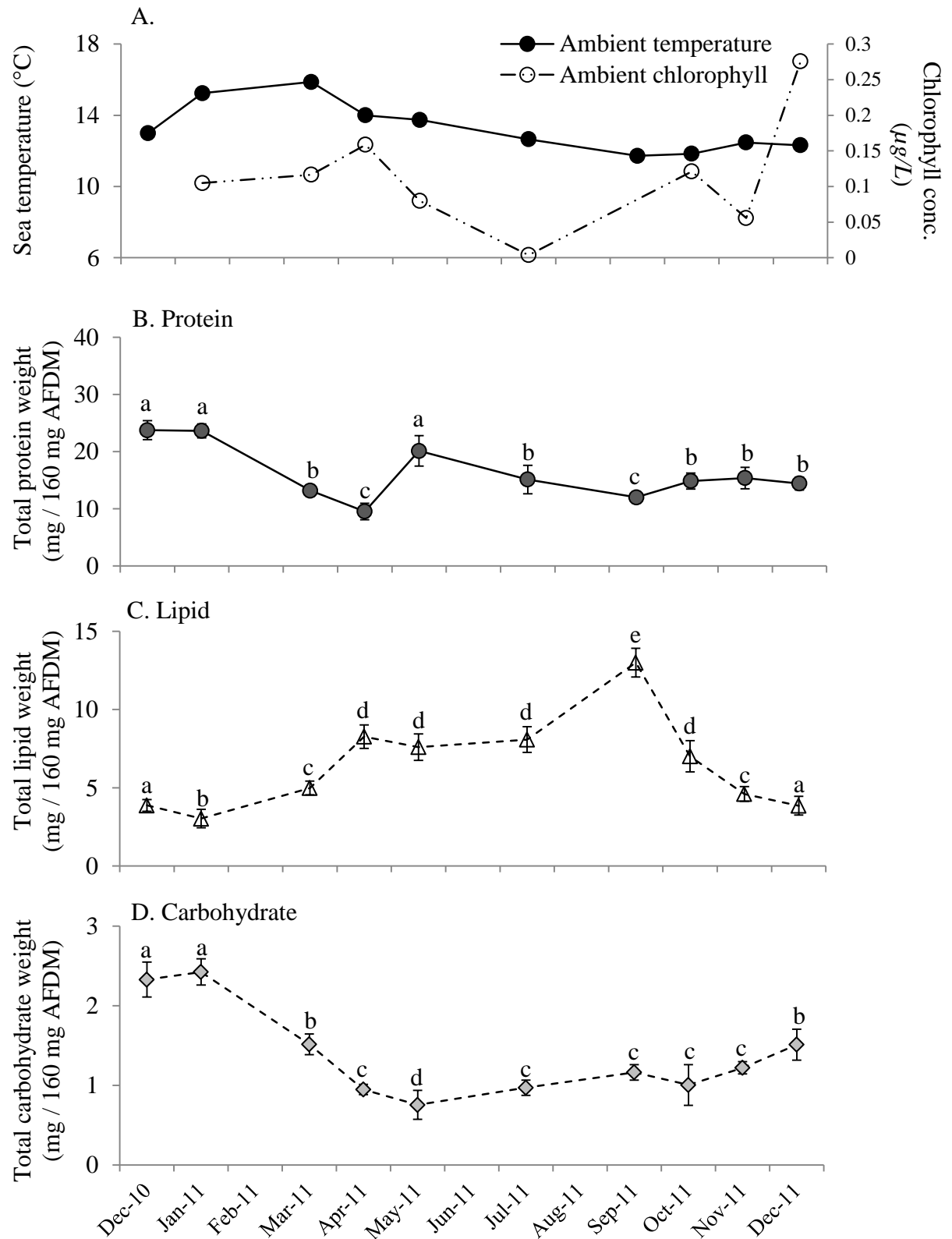
#### 3.3.1 Total seasonal biochemistry

##### *Soluble protein*

Total soluble protein levels within a 160 mg AFDM *Liothyrella neozelanica* individual showed the greatest amount of change (60%) throughout the sampling period, reaching a maximum of 23.7 mg in December 2010 and January 2011, before declining to a low of 9.5 mg in April 2011 (Fig. 3.2 B). Protein levels were significantly different from one another throughout the sampling period ( $F = 8.09$ ;  $df = 9, 99$ ;  $p < 0.0001$ ). A Tukey-Kramer comparison test revealed that the total soluble protein levels fluctuated between three major concentrations throughout the sampling period (Fig. 3.2 B). Total protein levels in December 2010, January 2011 and May 2011 were statistically similar, ranging from 23.7 mg to 20.1 mg, while total protein levels in April 2011 (9.5 mg) and September 2011 (12 mg) were also statistically similar. Total protein levels in the remaining months ranged between 15.4 mg and 13.2 mg and were not statistically different from one another (Fig. 3.2 B).

##### *Lipid*

Total lipid levels within a 160 mg AFDM *Liothyrella neozelanica* individual increased from December 2010 (3.9 mg) to reach a maximum of 13 mg in September 2011, before decreasing by 70% over October 2011 and November 2011 to 3.9 mg in December 2011 (Fig. 3.2 C). Total lipid levels were significantly different from one another throughout the sampling period ( $F = 17.58$ ;  $df = 9, 98$ ;  $p < 0.0001$ ). Total lipid levels in December 2010 and December 2011 were both 3.9 mg. Total lipid levels decreased significantly from December 2010 (3.9 mg) to 3 mg in January 2011, before increasing to 8.3 mg in April 2011 (Fig. 3.2 C). Total lipid levels remained statistically similar throughout April 2011 (8.3 mg) to July 2011 (8.1 mg) before increasing by 38% in September 2011 to reach a maximum concentration of 13 mg (Fig. 3.2 C).



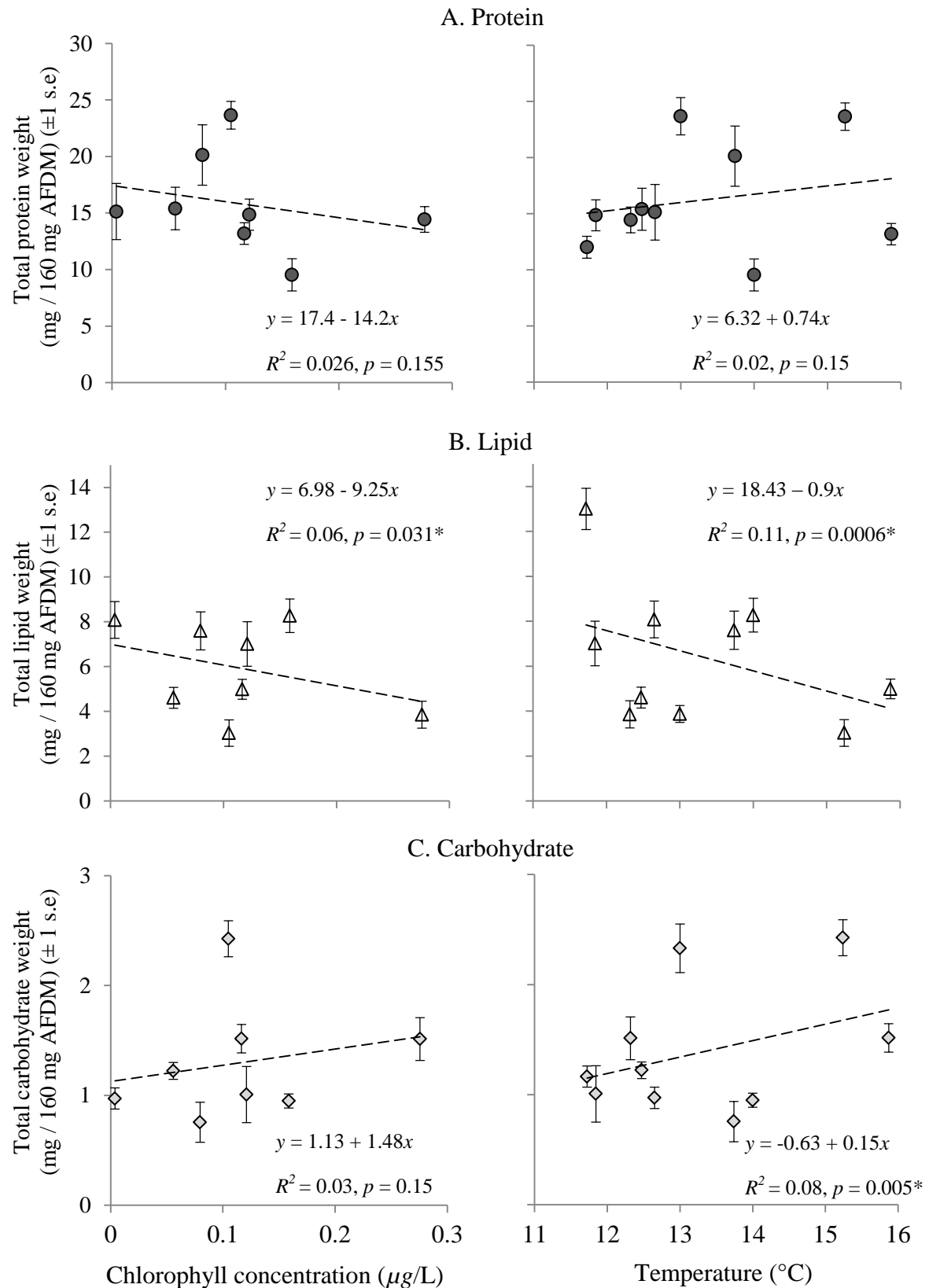
**Figure 3.2** Seasonal sea temperature (°C) and chlorophyll concentration (µg/L) (A) and total protein (B), lipid (C) and carbohydrate (D) levels for *L. neozelanica* within Tricky Cove (Different lower case letters denote significant difference among months,  $p < 0.05$ ) ( $n = 10$  / point  $\pm 1$  s.e) (Values expressed as weight of each component within a 160 mg ash-free dry mass (AFDM) individual) (N.b. Different scales for each biochemical component).

*Carbohydrate*

Total carbohydrate concentrations within a 160 mg AFDM *L. neozelanica* individual (0.8 – 2.4 mg), were in lower concentrations compared to levels of protein (9.5 – 23.7 mg) and lipid (3.0 – 13 mg) throughout the sampling period (Fig. 3.2 D). Total carbohydrate levels were found to be significantly different throughout the sampling period ( $F = 19.9$ ;  $df = 9, 98$ ;  $p < 0.0001$ ). Highest total carbohydrate concentrations occurred in December 2010 (2.3 mg) and January 2011 (2.4 mg), before declining to a low of 0.8 mg in May 2011 (Fig. 3.2 D). Total carbohydrate levels increased from May 2011 (0.8 mg) to July 2011 (1 mg) before remaining relatively constant throughout September 2011 (1.2 mg) to November 2011 (1.2 mg) (Fig. 3.2 D). Total carbohydrate levels increased in December 2011 (1.5 mg) to reach an identical level observed in March 2011 (1.5 mg) (Fig. 3.2 D).

*Environmental effects*

Total protein levels within a 160 mg AFDM individual, were relatively stable throughout the sampling period, despite changes in ambient sea temperature and ambient chlorophyll concentration (Fig. 3.2). A linear regression between total protein levels, ambient sea temperatures and ambient chlorophyll concentrations, revealed that ambient sea temperature ( $F = 2.15$ ;  $df = 9, 99$ ;  $p = 0.15$ ) and ambient chlorophyll concentration ( $F = 2.06$ ;  $df = 7, 79$ ;  $p = 0.16$ ) had no significant effect on total protein levels throughout the sampling period (Fig. 3.3). Linear regressions revealed total lipid levels were negatively correlated to ambient sea temperature ( $F = 12.6$ ;  $df = 9, 98$ ;  $p = 0.0006$ ) and ambient chlorophyll concentration ( $F = 4.825$ ;  $df = 7, 79$ ;  $p = 0.03$ ) (Fig. 3.3). A linear regression indicated total carbohydrate concentration to be significantly positively correlated to ambient sea temperature ( $F = 8.36$ ;  $df = 9, 98$ ;  $p = 0.005$ ), but not to ambient chlorophyll concentration ( $F = 2.08$ ;  $df = 7, 79$ ;  $p = 0.15$ ) (Fig. 3.3).



**Figure 3.3** Linear regressions of total *L. neozelanica* protein (mg / 160 mg AFDM) (A), lipid (mg / 160 mg AFDM) (B) and carbohydrate (mg / 160 mg AFDM) (C) vs. ambient chlorophyll concentration ( $\mu\text{g/L}$ ) and ambient sea temperature ( $^{\circ}\text{C}$ ) (Chlorophyll:  $n = 80$  / regression for all comparisons ( $\pm 1$  s.e.). Temperature:  $n = 100$  in protein regression ( $\pm 1$  s.e.),  $n = 99$  for lipid and carbohydrate regressions ( $\pm 1$  s.e.). \* denotes significant correlation).

### 3.3.2 Seasonal component biochemistry

#### 3.3.2 (a) *Lophophore*

##### *Protein*

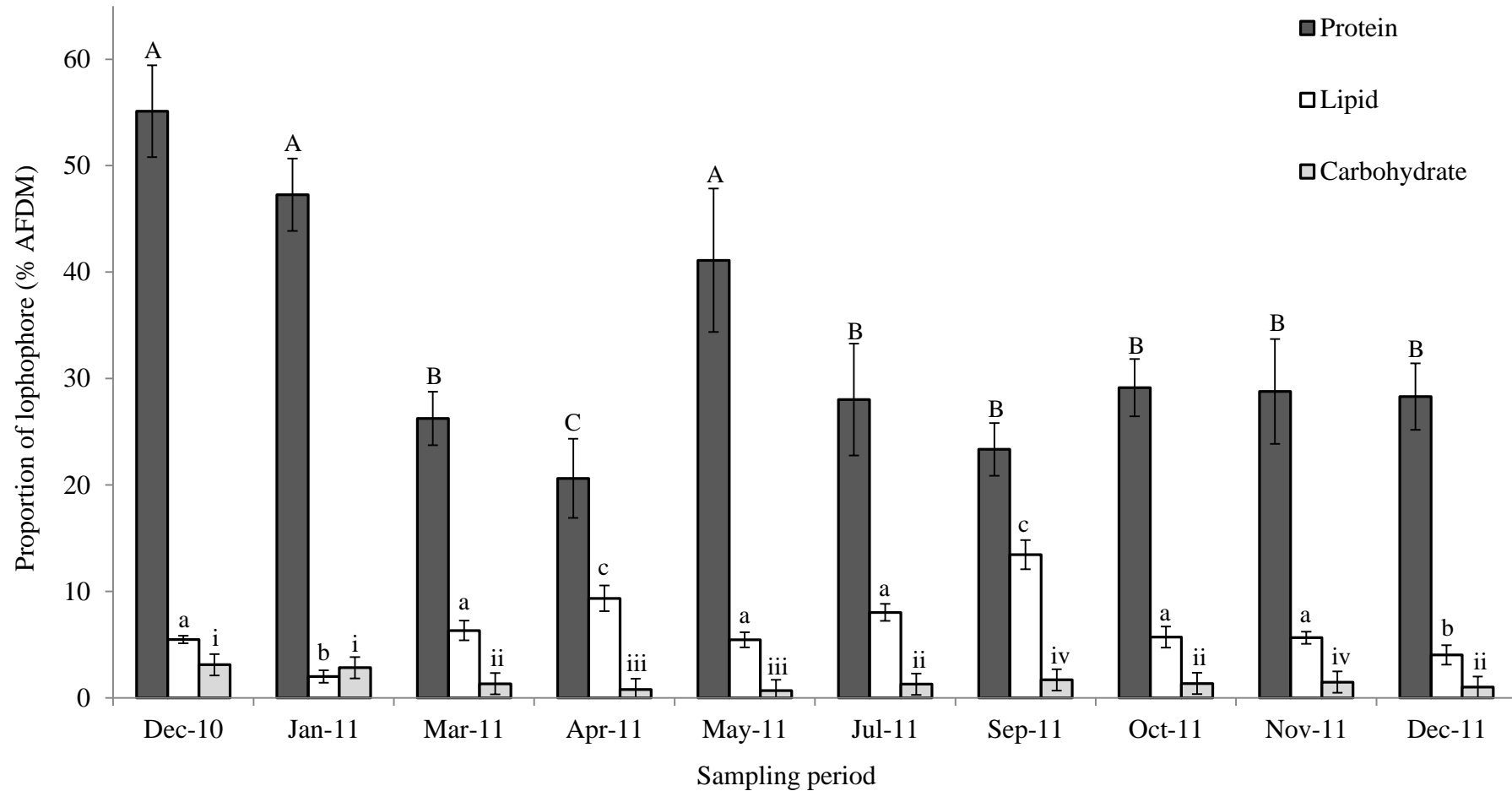
Protein was the dominant biochemical found within the lophophore throughout the sampling period making up 55.1% to 20.6% of the lophophore (Fig. 3.4). Protein levels ( $F = 7.34$ ;  $df = 9, 99$ ;  $p < 0.0001$ ), lipid levels ( $F = 10.87$ ;  $df = 9, 93$ ;  $p < 0.0001$ ) and carbohydrate levels ( $F = 25.7$ ;  $df = 9, 98$ ;  $p < 0.0001$ ) all showed significant variation within the lophophore throughout the sampled months (Fig. 3.4). Protein levels within the lophophore reached maximum concentrations in December 2010 ( $55.1 \pm 4.3\%$ ), January 2011 ( $47.3 \pm 3.4\%$ ) and May 2011 ( $41.1 \pm 6.8\%$ ), with no significant difference observed between these months (Fig. 3.4). Protein levels were statistically similar among the remaining months, with the exception of April 2011 ( $20.6 \pm 3.7\%$ ), which was significantly lower (Fig. 3.4).

##### *Lipid*

The amount of lipid within the lophophore was relatively insignificant compared to protein levels, with values ranging between 2% and 13.5% (Fig. 3.4). Lipid levels within the lophophore fluctuated over the sampling period reaching maximum values in April 2011 ( $9.3 \pm 1.2\%$ ) and September 2011 ( $13.5 \pm 1.4\%$ ). Lipid levels throughout the remaining months, were statistically similar ranging between 5.4% and 8%, with the exceptions of January 2011 ( $2 \pm 0.6\%$ ) and December 2011 ( $4 \pm 0.9\%$ ) which were significantly lower (Fig. 3.4).

##### *Carbohydrate*

Carbohydrate levels were 14 to 60 times lower than those of protein within the lophophore throughout the sampling period (Fig 3.4). Highest carbohydrate levels occurred in December 2010 ( $3.1 \pm 0.3\%$ ) and January 2011 ( $2.8 \pm 0.2\%$ ), before steadily declining to reach a minimum of  $0.7 \pm 0.2\%$  in May 2011. Carbohydrate levels between July 2011 and December 2011 were statistically different, but only ranged between 1% and 1.68% (Fig 3.4).



**Figure 3.4** Seasonal protein, lipid and carbohydrate proportions within the lophophore of *Liothyrella neozelanica* (Upper case letters represent significant difference in protein ( $n = 100$ ), lower case letters represent significant difference in lipid ( $n = 94$ ) and Roman numerals represent significant difference in carbohydrate ( $n = 99$ ) (Significant difference:  $p < 0.05$ ) (Each bar represents the mean  $\pm 1$  s.e).



### 3.3.2 (b) Stomach

#### *Protein*

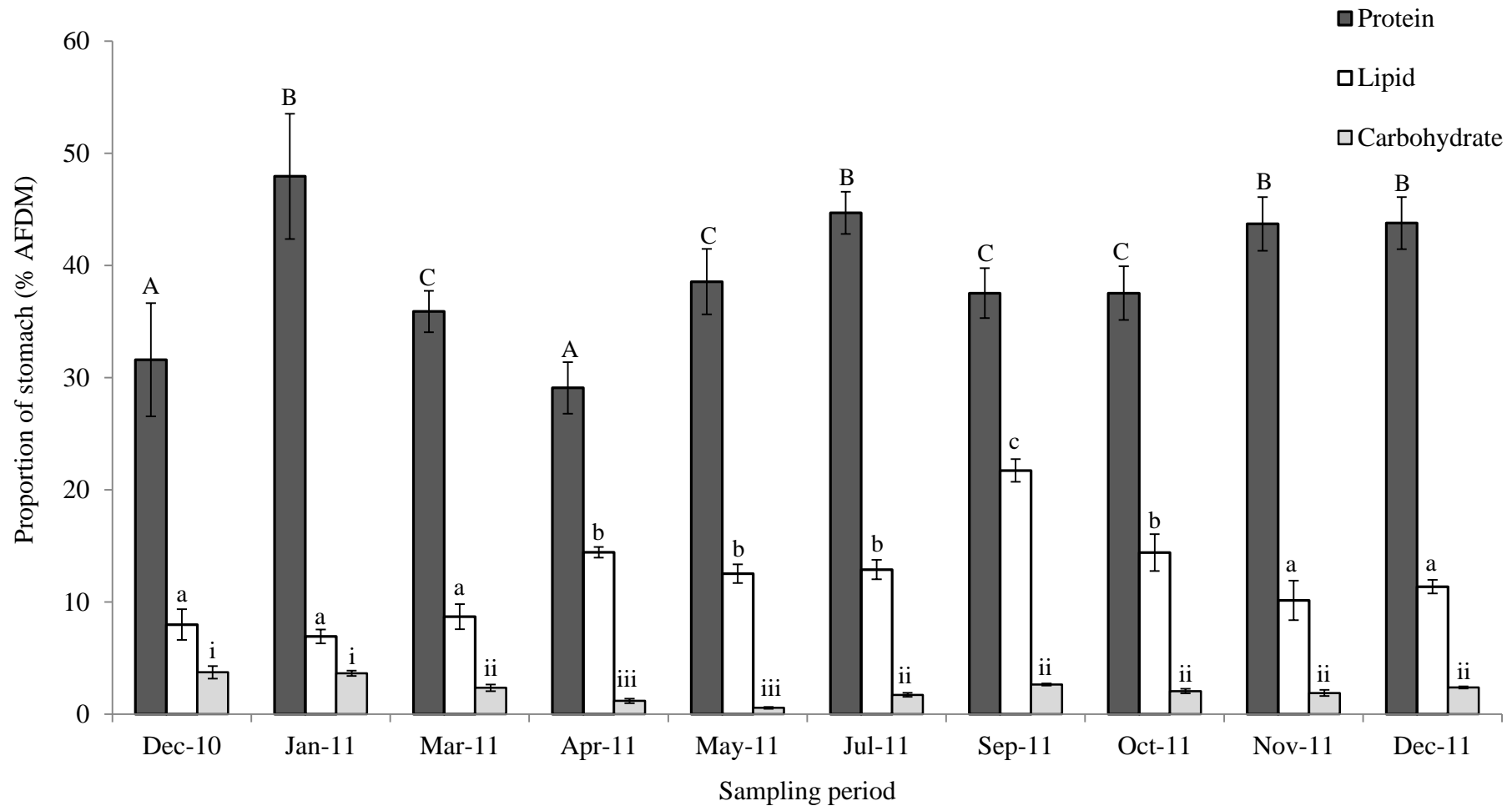
Soluble protein was the dominant biochemical found within the stomach of *L. neozelanica* throughout the sampled months (Fig. 3.5). Total protein ( $F = 4.76$ ;  $df = 9, 99$ ;  $p < 0.0001$ ), lipid levels ( $F = 15.23$ ;  $df = 9, 96$ ;  $p < 0.0001$ ) and carbohydrate levels ( $F = 24.97$ ,  $df = 9, 97$ ;  $p < 0.0001$ ) all varied significantly throughout the sampled months (Fig. 3.5). Total protein within the stomach remained relatively high throughout the sampling period, with highest protein levels occurring in January 2011 ( $48 \pm 1.8\%$ ), July 2011 ( $45 \pm 2.2\%$ ), November 2011 ( $44 \pm 2.3\%$ ) and December 2011 ( $44 \pm 1.5\%$ ) (Fig.3.5). Lowest protein levels occurred in December 2010 ( $32 \pm 5.6\%$ ) and April 2011 ( $29 \pm 2.9\%$ ). The remaining months had statistically similar protein levels ranging between  $36 \pm 2.3\%$  (March 2011) and  $39 \pm 1.9\%$  (May 2011) (Fig. 3.5).

#### *Lipid*

Total stomach lipid as a proportion of AFDM, was relatively consistent between December 2010 ( $8 \pm 0.6\%$ ) and March 2011 ( $9 \pm 0.5\%$ ), but increased by 40% to  $14 \pm 0.8\%$  in April 2011 (Fig. 3.5). Stomach lipid levels remained statistically similar throughout April 2011 ( $14 \pm 0.8\%$ ) and July 2011 ( $13 \pm 1\%$ ) before again increasing by 40% to reach a maximum lipid value of  $22 \pm 1.7\%$  in September 2011. After September 2011, lipid levels declined through October 2011 ( $14 \pm 1.8\%$ ) before reaching levels in November 2011 ( $10 \pm 0.6\%$ ) and December 2011 ( $11 \pm 1\%$ ) that were statistically similar to levels observed at the beginning of the sampling period (Fig. 3.5).

#### *Carbohydrate*

Throughout the sampling period total carbohydrate levels within the stomach ranged between 3.7 and 0.6% of AFDM, 8 to 68 times lower than those levels observed for protein (Fig. 3.5). Carbohydrate levels were highest in December 2010 ( $3.7 \pm 0.2\%$ ) and January 2011 ( $3.7 \pm 0.3\%$ ), before declining to a low of  $0.6 \pm 0.2\%$  in May 2011 (Fig. 3.5). Carbohydrate levels within the stomach remained statistically similar throughout the remaining sampled months, ranging between  $1.7 \pm 0.1\%$  (July 2011) and  $2.7 \pm 0.2\%$  (September 2011) (Fig. 3.5).



**Figure 3.5** Seasonal protein, lipid and carbohydrate proportions within the stomach of *Liothyrella neozelanica* (Upper case letters represent significant difference in protein ( $n = 100$ ), lower case letters represent significant difference in lipid ( $n = 97$ ) and Roman numerals represent significant difference in carbohydrate ( $n = 98$ ) (Significant difference:  $p < 0.05$ ) (Each bar represents the mean  $\pm 1$  s.e).

### 3.3.2 (c) *Gonad*

#### *Protein*

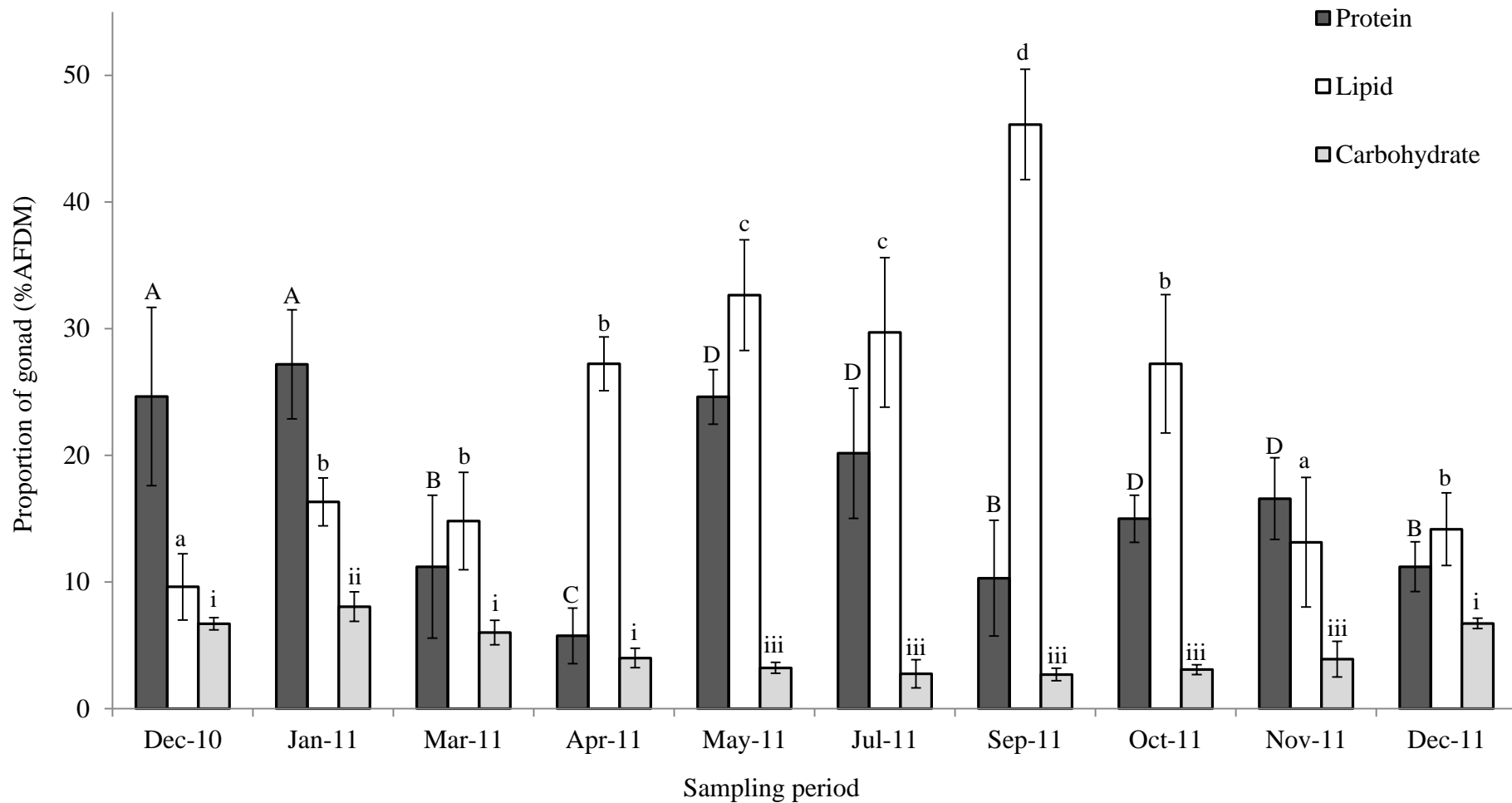
Throughout the sampling period, protein levels ( $F = 3.42$ ;  $df = 9, 87$ ;  $p < 0.0014$ ), lipid levels ( $F = 7.6$ ;  $df = 9, 96$ ;  $p < 0.0001$ ) and carbohydrate levels ( $F = 4.9$ ;  $df = 9, 98$ ;  $p < 0.0001$ ) all showed significant variation within the gonad of *L. neozelanica* (Fig. 3.6). Gonadal protein levels were highest in December 2010 ( $25 \pm 4.3\%$ ) and January 2011 ( $27 \pm 5.6\%$ ), before steadily declining to a low of  $5.7 \pm 2.2\%$  in April 2011 (Fig. 3.6). Protein levels increased four-fold from April 2011 to reach levels of  $24 \pm 5.1\%$  and  $20 \pm 4.6\%$  in May 2011 and July 2011 respectively. Protein levels decreased from July 2011 to levels ranging between  $10 \pm 1.9\%$  (September 2011) and  $17 \pm 2\%$  (November 2011) throughout the remaining sampled months (Fig. 3.6). Protein levels within the gonad were typically lower than those levels observed within the lophophore and stomach throughout the sampling period.

#### *Lipid*

Lipid showed the greatest change (9.6% to 46%) throughout the sampling period within the gonad of *L. neozelanica* (Fig. 3.6). Apart from December 2010, January 2011 and November 2011, lipid was the dominant biochemical constituent within the gonad, with levels reaching 1.3 to 4.5 times greater than those of protein (Fig. 3.6). In December 2010 the lowest lipid level ( $10 \pm 1.9\%$ ) within the gonad was recorded. The lipid level within the gonad steadily increased from December 2010 to reach a maximum of  $46 \pm 5.5\%$  in September 2011, before decreasing over the remaining sampled months (Fig. 3.6).

#### *Carbohydrate*

Carbohydrate levels were lower than those levels recorded for lipid and protein however, they were 1.1 to 6.7 times greater than carbohydrate levels observed in the lophophore and stomach (Fig 3.6). Carbohydrate levels within the gonad tended to follow an opposite trend to that shown by the lipid, with highest values (6% to 8%) occurring in the summer months (December – March), before steadily decreasing throughout the winter months (April – September) to a low of  $2.7 \pm 0.4\%$  (Fig. 3.6).



**Figure 3.6** Seasonal protein, lipid and carbohydrate proportions within the gonad of *Liothyrella neozelanica* (Upper case letters represent significant difference in protein ( $n = 87$ ), lower case letters represent significant difference in lipid ( $n = 97$ ) and Roman numerals represent significant difference in carbohydrate ( $n = 99$ ) (Significant difference:  $p < 0.05$ ) (Each bar represents the mean  $\pm 1$  s.e).

### 3.4 Discussion

#### 3.4.1 Total seasonal biochemistry

##### 3.4.1 (a) *Protein and carbohydrate*

Throughout the sampling period (December 2010 – December 2011) protein and carbohydrate levels within the internal tissues of *Liothyrella neozelanica* showed seasonal change. Greatest amounts of protein and carbohydrate occurred during summer months (December 2010 and January 2011), while lowest values occurred during the winter months of April 2011 and May 2011. This seasonal variation in protein and carbohydrate is consistent with previous studies involving temperate and polar bivalves, which show marked seasonality in biochemistry as a result of food supply, reproductive events and growth cycles (Ansell 1972, 1975; Beninger and Lucas 1984; Zandee *et al.* 1980; Brockington 2001; Ahn *et al.* 2003).

Throughout the sampled months protein was the major biochemical constituent within the internal tissues of *Liothyrella neozelanica*, with the exception of September 2011, when lipid levels (13 mg / 160 mg AFDM) surpassed protein (12 mg / 160 mg AFDM). Protein showed the greatest amount of variation throughout the sampling period (9.5 – 23.7 mg / 160mg AFDM), while carbohydrate levels stayed comparatively steady at low levels within the internal tissues of *L. neozelanica*. The temperate mussel *Mytilus edulis* utilises both protein and carbohydrate for energetics and growth, with carbohydrate levels reaching 10% of protein levels at the end of winter and increasing to around 70% of protein levels by the end of summer, signifying the importance of carbohydrate as an energy source (Gabbott 1983). In comparison to *Mytilus edulis*, the carbohydrate levels within *L. neozelanica* are much lower reaching 6% of protein levels at the end of winter and only 10 - 11.5% of protein levels at the end of summer. Due to the high levels of protein, high levels of protein variation and low levels of carbohydrate within the internal tissues of *L. neozelanica*, it can be assumed that protein is the major energy substrate utilised by *L. neozelanica*. Although occurring in relatively small amounts, carbohydrate levels within *L. neozelanica* still exhibit a seasonal cycle suggesting carbohydrate may be utilised as a secondary energy substrate in certain metabolic processes throughout an annual period. This is consistent with general knowledge involving biochemicals and animals described in Robbins (1983) and Hill *et al.* (2008).

Linear regressions showed that total protein levels were not significantly correlated with ambient sea temperature or ambient chlorophyll concentration throughout the sampling period. Furthermore, protein levels within *L. neozelanica* remained relatively stable throughout the sampling period despite seasonal variation in ambient chlorophyll concentration. A possible explanation may be that *L. neozelanica* utilises alternative energy sources other than phytoplankton.

From gut content analysis and feeding behaviour observations, McCammon (1969) showed that dissolved and colloidal organic nutrients within the surrounding water column are utilised by seven species of Rhynchonelliform brachiopods as an energy source. Mucus along the lophophore is thought to bind to dissolved organic nutrients within the water column and transport them through to the gut in high concentrations (McCammon 1969). Furthermore, McCammon (1969) noted that due to the nature of the blind ended gut within Rhynchonelliform brachiopods, it is more beneficial to utilise dissolved organic nutrients that produce little to no waste, than to digest phytoplankton which produces waste that needs to be transported out of the gut. McCammon (1969) concluded that dissolved and colloidal organic nutrients within the surrounding water column are the primary energy source of Rhynchonelliform brachiopods, supplemented marginally by phytoplankton. More recently, Peck *et al.* (2005) found that the Antarctic brachiopod *Liothyrella uva* exhibits a plastic diet, changing from phytoplankton as a primary food source in summer to re-suspended benthic diatoms as a primary food source in the winter. These findings by McCammon (1969) and Peck *et al.* (2005) are consistent with findings within this study which show that protein levels within *L. neozelanica* are independent of ambient chlorophyll concentration within Tricky Cove, New Zealand. It is therefore possible that *L. neozelanica* is an opportunistic feeder, relying primarily on dissolved organic nutrients and benthic diatoms, supplementing its biochemical stores throughout periods when phytoplankton levels increase. However, further research of ambient food concentrations and a dietary analysis of *L. neozelanica* are required in order to fully clarify this.

There was significant positive correlation between carbohydrate and ambient sea temperature throughout the sampling period. The increase in carbohydrate with increasing sea temperature is consistent with findings for *Mytilus edulis*, where carbohydrate levels significantly increase over summer months as temperature and chlorophyll levels increase (Gabbott 1983). Despite

the linear regression between ambient chlorophyll concentration and carbohydrate showing no significant correlation within this study, a positive trend was observed whereby carbohydrate levels increased as chlorophyll concentrations increased. Therefore carbohydrate levels, although minor within the tissues of *L. neozelanica*, may reflect the seasonality in phytoplankton and sea temperature within Tricky Cove throughout the sampling period.

### 3.4.1 (b) *Lipid*

The total amount of lipid within the internal tissues of *L. neozelanica* exhibited a strong trend throughout the sampling period, steadily increasing throughout the year to reach a maximum in September 2011, before steadily decreasing to previous levels. Lipid levels within marine invertebrates have been well described, particularly in bivalve species (Ansell 1972, 1974, 1975; Taylor and Venn 1979, Zandee *et al.* 1980, Beninger and Lucas 1984). Lipid levels have been found to reflect reproductive cycles in the majority of marine invertebrates, reaching maximum values during spawning periods as a result of high levels of lipid content within oocytes (Beninger and Lucas 1984; Gabbott 1983). Lipid levels in this study increased over winter despite decreasing sea temperature and ambient chlorophyll concentration, suggesting it is not utilised for energy, but stored and involved in gametogenesis in *L. neozelanica*. Interestingly, carbohydrate levels decrease between January 2011 and April 2011 as lipid levels steadily increase, possibly due to carbohydrate stores being converted to lipid to be used in gametogenesis. Conversion of carbohydrate stores to gamete lipids is common in marine invertebrates, including several species of temperate clam (*Tapes decussatus* and *Tapes philippinarum*) and the temperate scallop *Chlamys opercularis* (Taylor and Venn 1979; Beninger and Lucas 1984).

Throughout the sampling period total lipid content within *L. neozelanica* was found to be negatively correlated with both ambient sea temperature and ambient chlorophyll concentration. This may be explained by the storage of lipid throughout winter in preparation for spawning in spring, while temperature and chlorophyll levels reach minimum values. Following spawning, lipid levels drop as temperature and chlorophyll levels increase over the summer months. Such inverse relationships between lipid level and environmental conditions have also been observed in the Antarctic brachiopod *Liothyrella uva* (Peck *et al.* 1987).

### 3.4.2 Component seasonal biochemistry

#### 3.4.2 (a) *Lophophore*

Within the lophophore, protein was observed to be the major biochemical substrate. The lophophore serves several purposes, acting as a feeding organ, brooding larvae and also playing an important role in metabolism, serving in both respiration and excretion (Rudwick 1970; Richardson 1986). The high levels of protein found within the lophophore in comparison to lipid and carbohydrate levels is most likely a direct result of the activities that the lophophore is involved in, with protein being used as the main energy source to drive metabolism and feeding. Interestingly protein levels within the lophophore were found to decrease over the months of March 2011 and April 2011, while lipid levels increased not only within the lophophore, but also within the gonads of *L. neozelanica*. Moreover, lipid levels within the lophophore appeared to follow an inverse relationship with protein, with higher lipid levels occurring as protein levels dropped and lower lipid levels as protein levels increased. These results suggest that protein reserves within the lophophore may be being converted to lipid and transferred to the gonads as gamete development begins. This conversion and transfer of lipid has also been seen in marine molluscs, including the queen scallop *C. opercularis*, where glycogen is often converted to lipid in certain muscle types and transferred to the gonad during reproductive development (Taylor and Venn 1979).

Carbohydrate levels within the lophophore ranged from 0.7% to 3% throughout the sampled months which was 14 to 60 times lower than protein levels. Such low levels of carbohydrate within the lophophore suggest that it may be a minor metabolic substrate, with protein and lipid acting as the major energy substrates.

#### 3.4.2 (b) *Stomach*

Protein was the dominant biochemical substrate occurring in the stomach of *L. neozelanica* throughout the sampled months. High protein levels within the stomach suggest that protein is the main energy storage product of *L. neozelanica*, but may also reflect a stable high protein dietary intake throughout the sampling period, despite changing ambient chlorophyll concentrations. This would again agree with those results found by McCammon (1969),



suggesting that dissolved organic nutrients are the primary food of Rhynchonelliform brachiopods as opposed to phytoplankton (Refer Section 3.4.1 (a)).

As seen in the lophophore, protein appears to be negatively related to lipid with increased lipid levels coinciding with decreased protein levels between months. The relationship between protein and lipid appears less pronounced in the stomach when compared to the lophophore; however the changes are still substantial with protein levels dropping by 16% between July 2011 and September 2011, while lipid levels increased by 40%. This relationship, like that in the lophophore, suggests that protein may be being converted into lipid and transferred into the gonad to aid in reproductive development. Carbohydrate levels in the stomach were low in respect to protein and lipid and were relatively stable throughout the study period. Unlike many bivalves, such as the common *Mytilus edulis*, that utilise both carbohydrate and protein as main energy substrates (Ansell 1972; Zandee *et al.* 1980; Gabbott 1983), *L. neozelanica* appears to rely heavily on protein as its major energy substrate, while carbohydrate appears to be used for specific roles that require relatively low quantities.

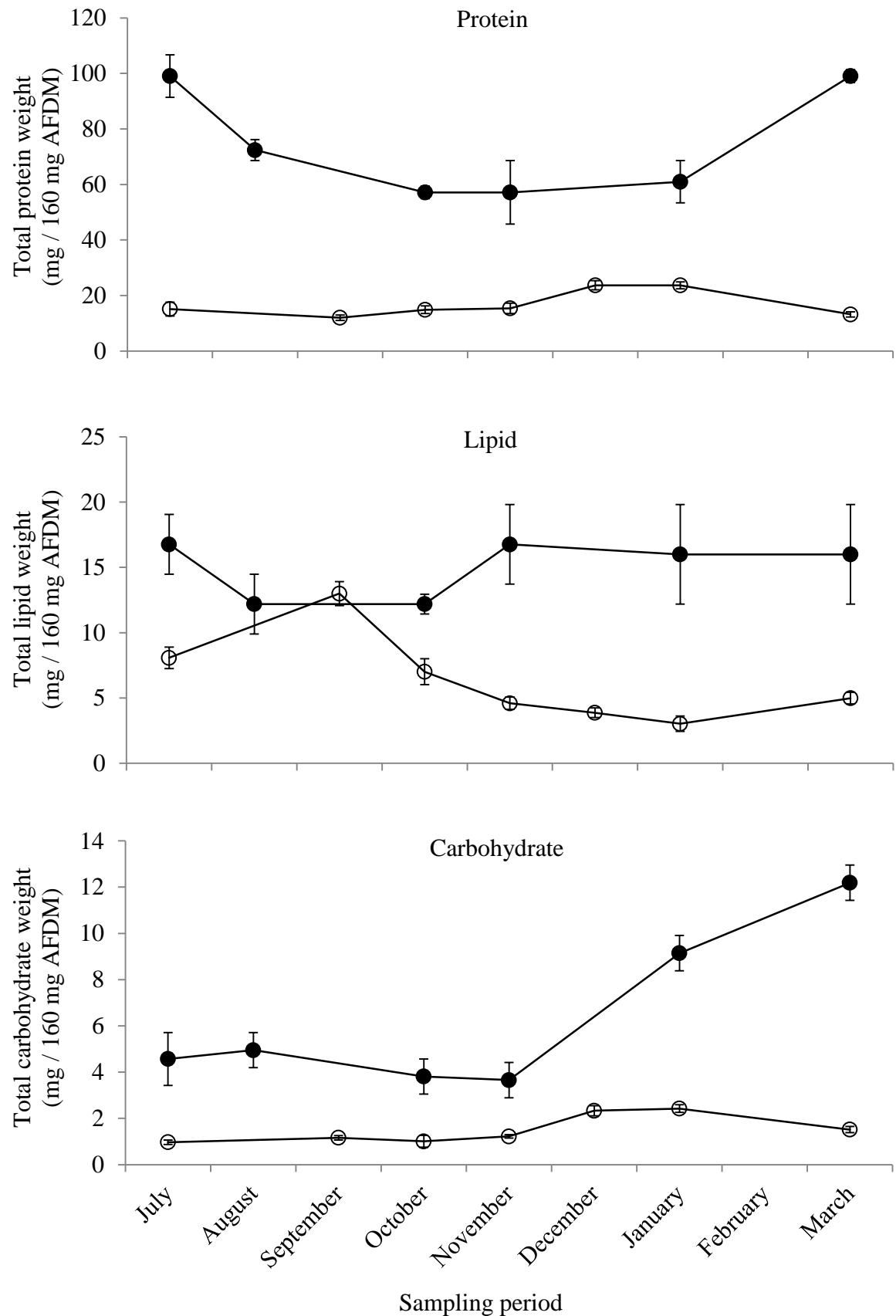
### 3.4.2 (c) *Gonad*

The gonad of *L. neozelanica* was dominated by lipid throughout most of the sampling period with the exceptions of December 2010 and January 2011, when protein was 2.6 and 1.7 times higher respectively. Lipid levels steadily increased, peaking in September 2011 before steadily declining to previous levels throughout the remaining sampled months. The common temperate mussel *Mytilus edulis* has been shown to conserve lipids throughout autumn until spring, utilising it in gametogenesis (Gabbott 1983). Furthermore, a significant reduction in lipid levels, as seen after September 2011 in *L. neozelanica*, has been linked to spawning events in other marine invertebrates including temperate bivalves and the Antarctic brachiopod *Liothyrella uva* (Gabbott 1983; Peck *et al.* 1987). Therefore it is reasonable to suggest that *L. neozelanica* stores lipid within its gonads throughout autumn and winter for use in gametogenesis and spawns during spring; signified by a considerable reduction in lipid content within the gonad.

Both protein and carbohydrate levels fluctuated within the gonad, with highest values observed during the summer months (December 2010 to January 2011) when lipid levels were lowest. Again, as seen in the lophophore and the stomach, protein and carbohydrate levels declined as lipid levels increased within the gonad, suggesting that carbohydrate and protein may be being converted into lipid to be used in gametogenesis throughout the winter months. The variability in protein levels throughout the sampled months and the low levels of carbohydrate within the gonad, suggest that protein is utilised as the major energy substrate within the gonadal tissues of *L. neozelanica*, whereas lipid is utilised more predominately in gametogenesis.

### 3.4.3 *Liothyrella* comparisons

Protein, lipid and carbohydrate comparisons between the Antarctic *Liothyrella uva* and the temperate *Liothyrella neozelanica* are shown in Figure 3.7. Protein levels within the internal tissues of *L. uva* are between 2.6 and 7.5 times higher than levels observed within the internal tissues of *L. neozelanica*. Furthermore, the levels of protein and carbohydrate within the tissues of *L. uva* show a marked seasonal trend with decreased levels in winter and increased levels throughout summer. This seasonality of protein and carbohydrate within *L. uva* is thought to be a direct consequence of seasonal food availability within the Southern Ocean (Peck *et al.* 1987). Due to food availability rapidly declining throughout winter in the Southern Ocean, Antarctic marine invertebrates including *L. uva*, rely on biochemical stores that are accumulated throughout the summer months when primary productivity is high (Peck *et al.* 1986, 1987; Ahn *et al.* 2003). Protein and carbohydrate levels within *L. neozelanica* show a seasonal trend, reaching lowest levels in the winter and highest levels in the summer; however, the amount of seasonal change exhibited by *L. neozelanica* is far less than that observed in *L. uva*. The differences in protein / carbohydrate levels and differences in the magnitude of seasonal change between *L. uva* and *L. neozelanica*, suggest that *L. uva* stores far higher levels of biochemicals within its tissues in order to over-winter, whereas *L. neozelanica* may have adequate seasonal food supply within Tricky Cove and therefore does not need to store large quantities of energy substrate. Interestingly, carbohydrate levels within *L. uva* increase 3-fold between winter and summer, reaching levels 2.5 times higher than those found in *L. neozelanica*. This may indicate that carbohydrate holds more importance as an energy substrate within *L. uva* than observed in *L. neozelanica*.



**Figure 3.7** Seasonal biochemical comparisons between *Liothyrella uva* (●) (Peck *et al.* 1987) and *Liothyrella neozelanica* (○). Values are averages (±1 s.e) based on a 160 mg AFDM individual.

Lipid levels within both *L. neozelanica* and *L. uva* showed a marked drop between August and October, suggesting that these two species spawn throughout spring. Within the Southern Ocean, invertebrates often spawn at the beginning of spring so that larval release coincides with increased levels of food availability (Pearse *et al.* 1991). Interestingly, lipid levels are higher within the internal tissues of *L. uva* in comparison to *L. neozelanica*, suggesting that *L. uva* may utilise lipid in gametogenesis, but also as an energy storage substrate, to be utilised overwinter. Furthermore, lipid levels within *L. uva* rapidly recover post-spawning to reach pre-spawning levels within one month, whereas lipid levels within *L. neozelanica* steadily decline before rising slightly at the end of summer. This rapid recovery in lipid may be an adaptation by *L. uva* to rapidly increase stores throughout the summer while food availability is high in preparation for the food-limited winter months.

### 3.4.4 Conclusion

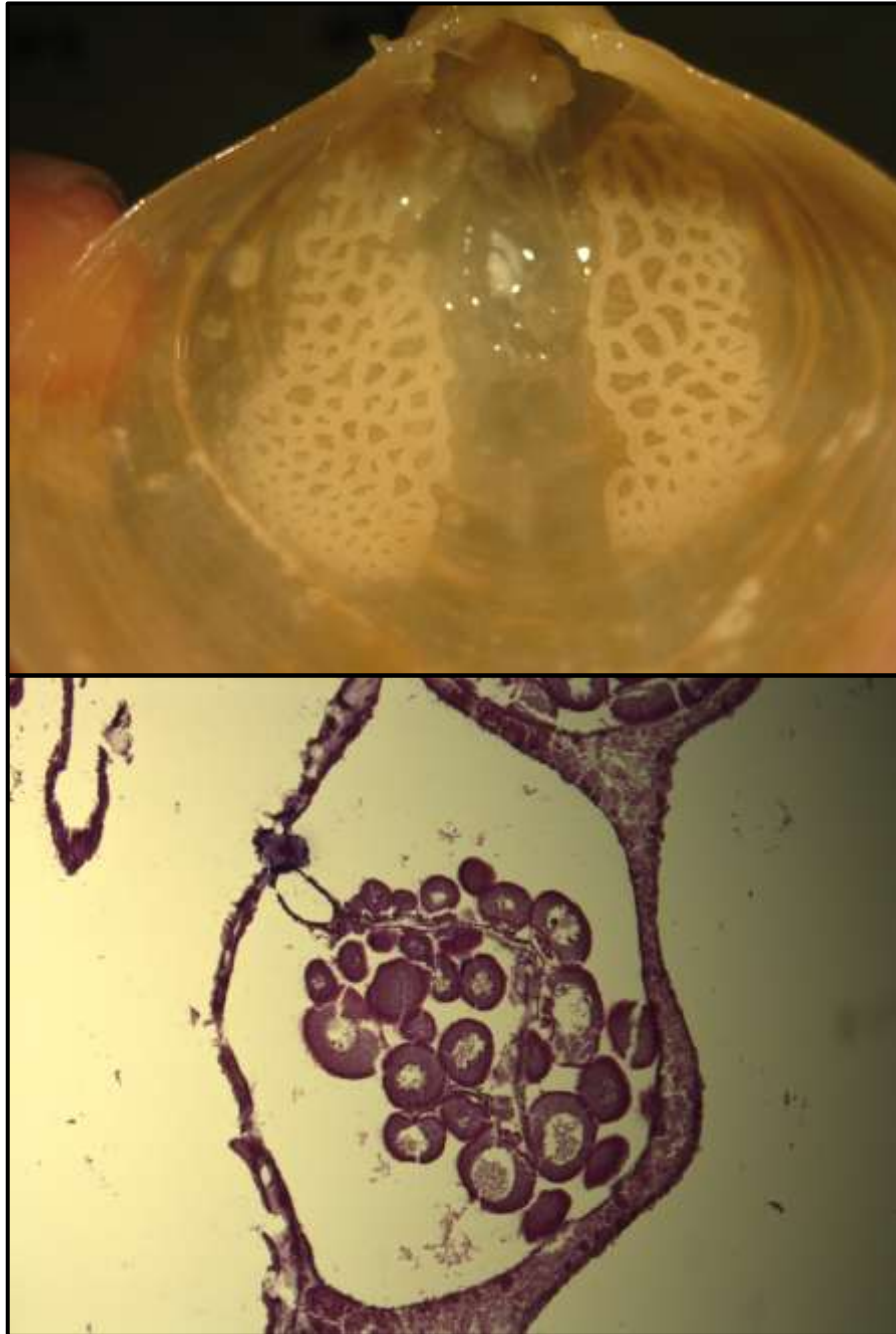
Total protein levels within *L. neozelanica* showed the greatest amount of variation throughout the sampling period and occurred in the highest levels within the internal tissues, suggesting it is utilised as the main energy substrate. Carbohydrate levels occurred in relatively small amounts within the internal tissues of *L. neozelanica* suggesting that it is a secondary energy substrate, utilised in minor quantities for specific metabolic processes. Total lipid levels within *L. neozelanica* increased steadily across winter, before dropping rapidly in spring, possibly suggesting a spawning event. Protein and carbohydrate levels within each internal component of *L. neozelanica* were found to be inversely related to lipid levels, suggesting the possible conversion and transfer of protein and carbohydrate into lipid to be used in gametogenesis.

Protein, lipid and carbohydrate levels within the internal tissues of *L. uva* were all higher than those levels found within *L. neozelanica*, suggesting *L. uva* may store larger quantities of each biochemical within its tissue as an adaptation to the extreme seasonality in food availability within the Southern Ocean. A coincident reduction in lipid levels during spring, suggests that both *L. uva* and *L. neozelanica* may spawn at this time, triggered by warming sea temperature and increased food availability.



# CHAPTER 4

## REPRODUCTIVE CYCLE OF *LIOTHYRELLA NEOZELANICA*



Top: *Liothyrella neozelanica* female gonads. Bottom: Histology slide of female *L. neozelanica* gonad.

**“I don’t think this is safe yet for human reproduction” (Michael West).**

## 4.1 Introduction

The reproduction of brachiopods is not well defined, with the majority of research describing gametogenesis and larval development. Moreover, little information is available on factors that affect these reproductive processes except that parasitism was found to reduce / destroy gonads within *Glottidia pyramidata* and *Liothyrella neozelanica* (Paine 1962; Long and Stricker 1991; Chuang 1994). Furthermore, the majority of reports on brachiopod reproduction are laboratory based, with some studies holding brachiopods in artificial conditions that varied substantially from ambient conditions in their habitats during the time of collection (Long 1964; Chuang 1959; Long and Stricker 1991). While these studies have provided vital insight into the reproductive and larval development of brachiopods, it is still unclear what environmental factors influence the reproductive cycle in the majority of brachiopod species.

Reproductive cycles within marine invertebrates have been found to be affected by a range of exogenous and endogenous factors (Mackie 1984). Of the external factors, temperature has been described as the main driver of gamete maturation and spawning in marine invertebrates, in particular bivalves such as *Mytilus edulis* (Mackie 1984). Annual fluctuations in temperature and temperature thresholds have been shown to trigger bivalve spawning events, as shown in *Mytilus edulis*, which initiates spawning when sea temperatures exceed 10°C to 12°C (Mackie 1984). Other external factors including lunar periodicity, depth, food availability, salinity, and population density have all been shown to be linked to spawning in marine invertebrates along with endogenous factors such as neurosecretions and hormonal control (Nelson 1936; Sastry 1979; Mackie 1984).

The reproduction of New Zealand brachiopods is largely undefined with only a few papers by Tortell (1981), Hoverd (1984) and Chuang (1994) describing broad spawning times and larval development for a small number of species (Tortell 1981; Hoverd 1984; Chuang 1994). Reproductive development, larval development and spawning observations have all been described for *Liothyrella neozelanica* (Tortell 1981; Chuang 1994). Tortell (1981) observed that 7 out of 8 female *L. neozelanica* brachiopods had partly spawned between February and March, with only one female having a full, ripe gonad. However, Tortell noted that the *L. neozelanica* individuals observed had undergone autolysis and were not fresh, being out of

water for over 24 hours. Chuang (1996) observed spawning by *L. neozelanica* individuals in laboratory conditions during February and as a consequence, it has been suggested that *L. neozelanica* spawns throughout February to March. Interestingly Lee *et al.* (2010) observed from settling plate recruitment, that *L. neozelanica* showed possible evidence of spawning between February and March, but also evidence of a second spawning episode occurring in spring (September - November). Multiple spawning episodes are not uncommon amongst brachiopods with the New Zealand Rhynchonelliform brachiopod *Calloria inconspicua* being found to spawn twice (April and August) throughout an annual period (Doherty 1979).

Findings from previous studies (Tortell 1981; Chuang 1994, 1996; Lee *et al.* 2010) have shown that the seasonal reproductive cycle of *L. neozelanica* is still relatively undefined and further research is needed to clarify this process. Furthermore, there is little information on factors, both exogenous and endogenous, that may be responsible for controlling reproductive cycles within New Zealand brachiopods and brachiopods in general. In order to fully understand New Zealand brachiopod communities and their relative abundance throughout New Zealand waters, it is important to define their reproductive strategies to provide insight into recruitment rates and population stability. Moreover, by addressing the effects of environmental factors such as temperature, photoperiod and food availability on *L. neozelanica*'s reproductive cycle, insight into how increased sea temperature resulting from climate change may affect spawning cycles of brachiopods can be achieved.

#### 4.1.1 Chapter objective

1. The objective of this study is to determine the seasonal reproductive cycle of *Liothyrella neozelanica*. In order to achieve this objective the following aims will be addressed:

- Determine the spawning time/s of *Liothyrella neozelanica*
- Determine the seasonal gametogenic development of *Liothyrella neozelanica*
- Determine the influence of ambient temperature, photoperiod and chlorophyll concentration on the reproductive cycle of *Liothyrella neozelanica*



## 4.2 Methods

### 4.2.1 Environmental factors

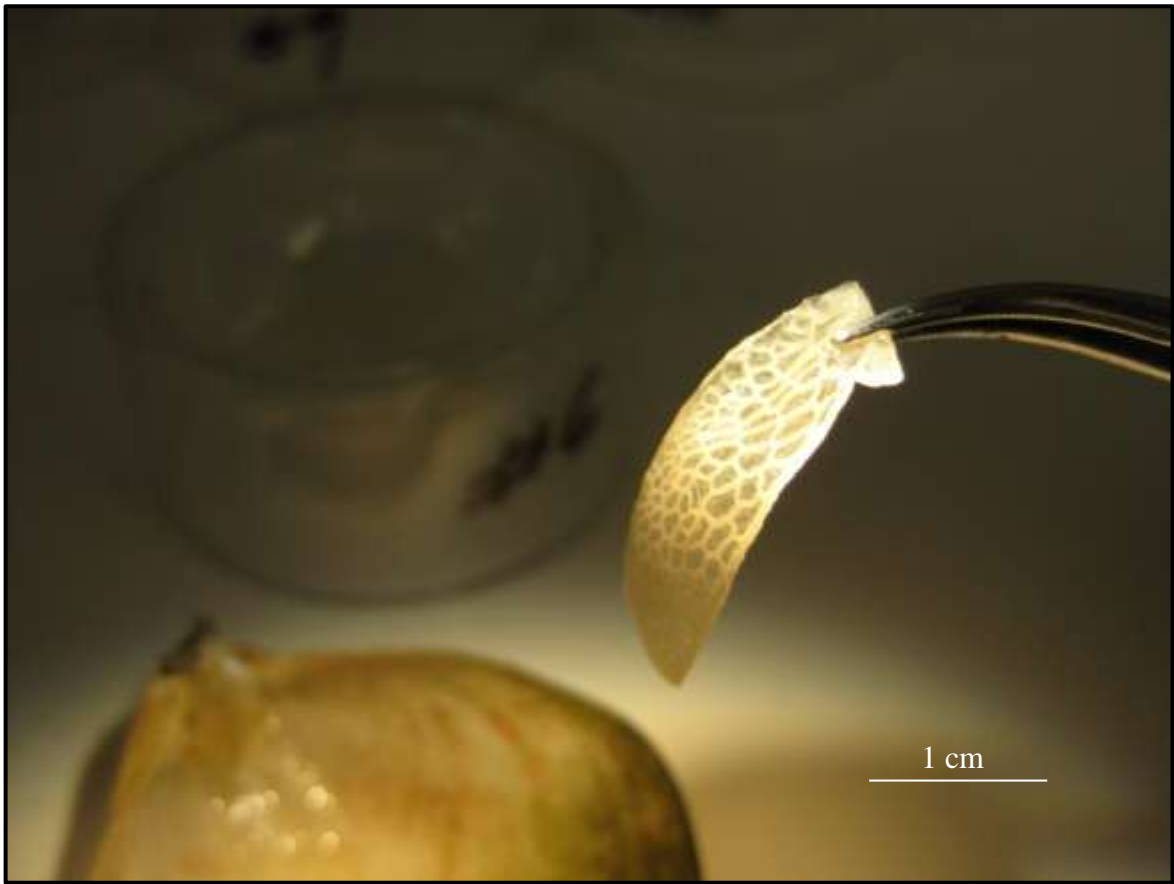
Ambient sea temperature (°C), photoperiod (hours) and chlorophyll concentration ( $\mu\text{g} / \text{L}$ ) were recorded in Tricky Cove throughout the sampling period (Refer Chap. 2, Sec. 2.2.1).

### 4.2.2 Gonad lipid levels

Seasonal lipid levels within the gonads of *Liothyrella neozelanica* were established using techniques described in Chapter 3, section 3.2.3. Gonad lipid levels were expressed as the proportion (%) of lipid within the gonads (AFDM) of *L. neozelanica*.

### 4.2.3 Gonad Index

Over the course of the sampling period (December 2010 to December 2011), *L. neozelanica* brachiopods were collected from Tricky Cove and transported back to PML (Refer Chap. 2, section 2.2.1). On arrival at PML (< 24 hours), 10 *L. neozelanica* individuals had their gonads removed (Fig. 4.1). The wet weight (mg) of the gonads and remaining tissue/shells were recorded. The components were dried in a 60°C oven for 48 hours and then ashed at 470°C for 12 hours. The ash-free dry mass (AFDM) of each gonad and corresponding shell was recorded. The proportion of gonad AFDM (mg) to the overall body AFDM (mg) was calculated for each individual (%), averaged over each collection, and used to create a seasonal gonad index (G.I.) for *L. neozelanica*.



**Figure 4.1** Dissected female *Liothyrella neozelanica* gonad.

#### 4.2.4 Histology

After each collection, the gonads of 10 *L. neozelanica* individuals were dissected out and stored in seawater buffered formalin (10%). Gonadal tissues were dehydrated and embedded in wax following methods described by Humason (1981), using an Elliott tissue processor. Samples were sectioned (7  $\mu\text{m}$  thick), mounted and stained with a haematoxylin / eosin staining technique in order to differentiate nuclei from cytoplasmic structures. Histological slides were used to establish the sex of each animal and to determine the gametogenic stage based on the development of the genital lamella and the appearance of associated oocytes. Oocyte development was divided into four generic stages based on previous studies involving *Terebratulina retusa* (James *et al.* 1991b) and *Terebratella sanguinea* (Ostrow 2007). Stage 1 (undeveloped gonad) was defined as the presence of clustered small oocytes with no visible nucleus. Stage 2 was defined by the development of a nucleus in the majority of oocytes (developing gonad). Stage 3 was defined as the presence of high numbers of mature oocytes, detached from the lamella and filling the majority of the lumen (mature gonad). The final

stage (4) was defined as the presence of necrotic / degrading tissue and the absence of large numbers of mature oocytes (post-spawning).

Average oocyte density, oocyte size and the proportion of sperm occupying the lumen (sperm proportion), were determined from the slides of each collection to establish the seasonal reproductive cycle of *L. neozelanica*. Average oocyte density was determined as the number of oocytes occurring within the area of the lumen (mm<sup>2</sup>). Oocyte size was determined by measuring the surface area of each oocyte (μm<sup>2</sup>) as opposed to measuring the diameter, as oocytes are often irregular in shape. Average sperm proportion was determined as the area of the lumen occupied by spermatozoa (%). Oocyte density, oocyte size and sperm proportion were averaged across all samples for each collection.

The seasonal ambient sea temperature, photoperiod, chlorophyll concentration and gonad lipid proportion were related to the seasonal averages of oocyte density, oocyte size and sperm proportion, to distinguish the relationships and influence each factor had on the seasonal gametogenic development of *L. neozelanica*.

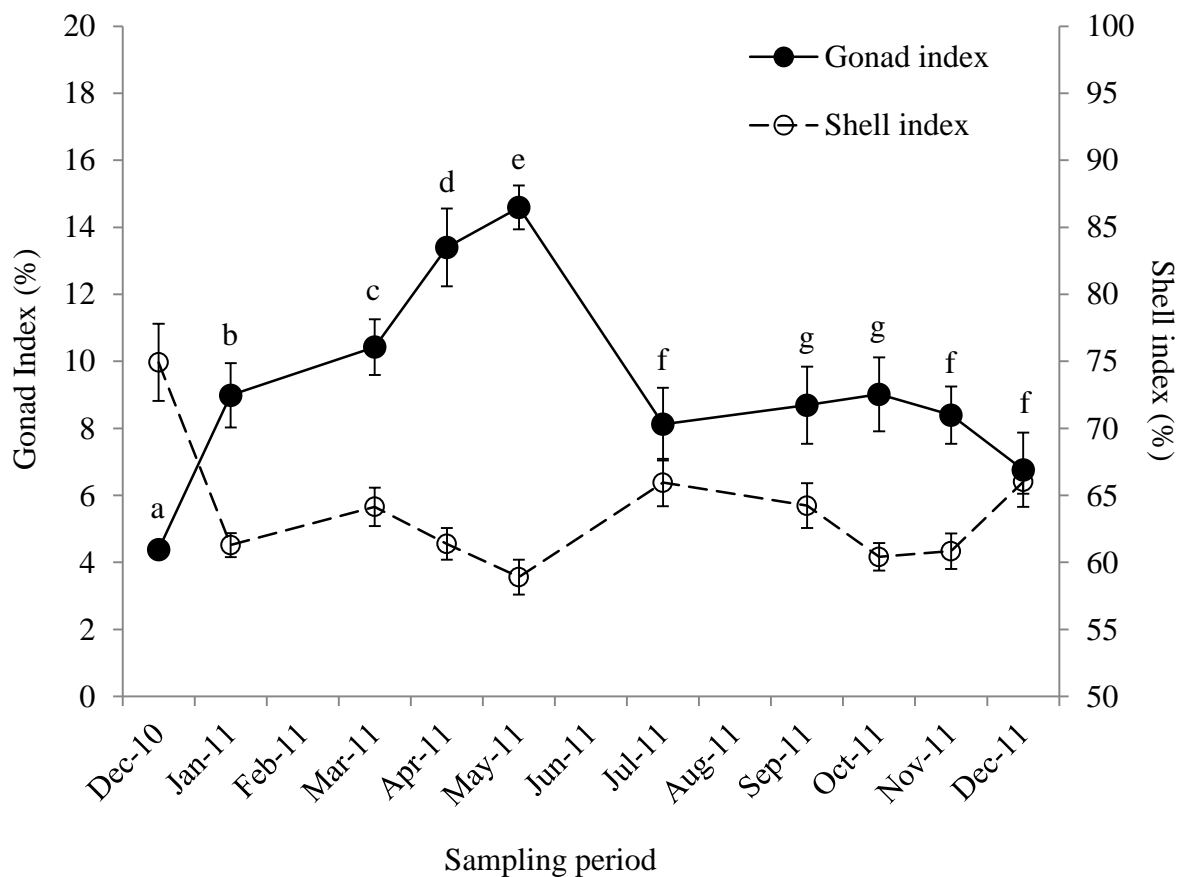
#### **4.2.5 Statistical analysis and image processing**

The image processing software Image J was used to establish sperm proportion, oocyte size and oocyte densities within *L. neozelanica*. Statistical analyses were carried out using the JMP 7 statistical package. One –Way ANOVAs were used to determine the seasonal differences in G.I, sperm proportion, oocyte size and oocyte density. Tukey – Kramer comparisons were used to specify differences within each ANOVA. Linear regressions were used to investigate relationships / effects ambient sea temperature, photoperiod, chlorophyll concentration and gonad lipid proportion had with seasonal oocyte density, oocyte size and sperm proportion of *L. neozelanica*.

### 4.3 Results

#### 4.3.1 Gonad Index

The gonad index (ratio of gonad AFDM to body AFDM) of *Liothyrella neozelanica* varied significantly among sampled months ( $F = 8.86$ ;  $df = 9, 116$ ;  $p < 0.0001$ ) (Fig. 4.2). Gonad proportions steadily increased 3-fold between December 2010 (4.4%) and May 2011 (14.6%), before decreasing to 8.1% in July 2011 (Fig. 4.2). The gonad index remained relatively stable throughout September 2011 (8.7%) and October 2011 (9%), but significantly declined through November 2011 (8.4%) and December 2011 (6.8%) (Fig. 4.2). The gonad index increased and decreased as the shell index (%) decreased and increased respectively (Fig 4.2).



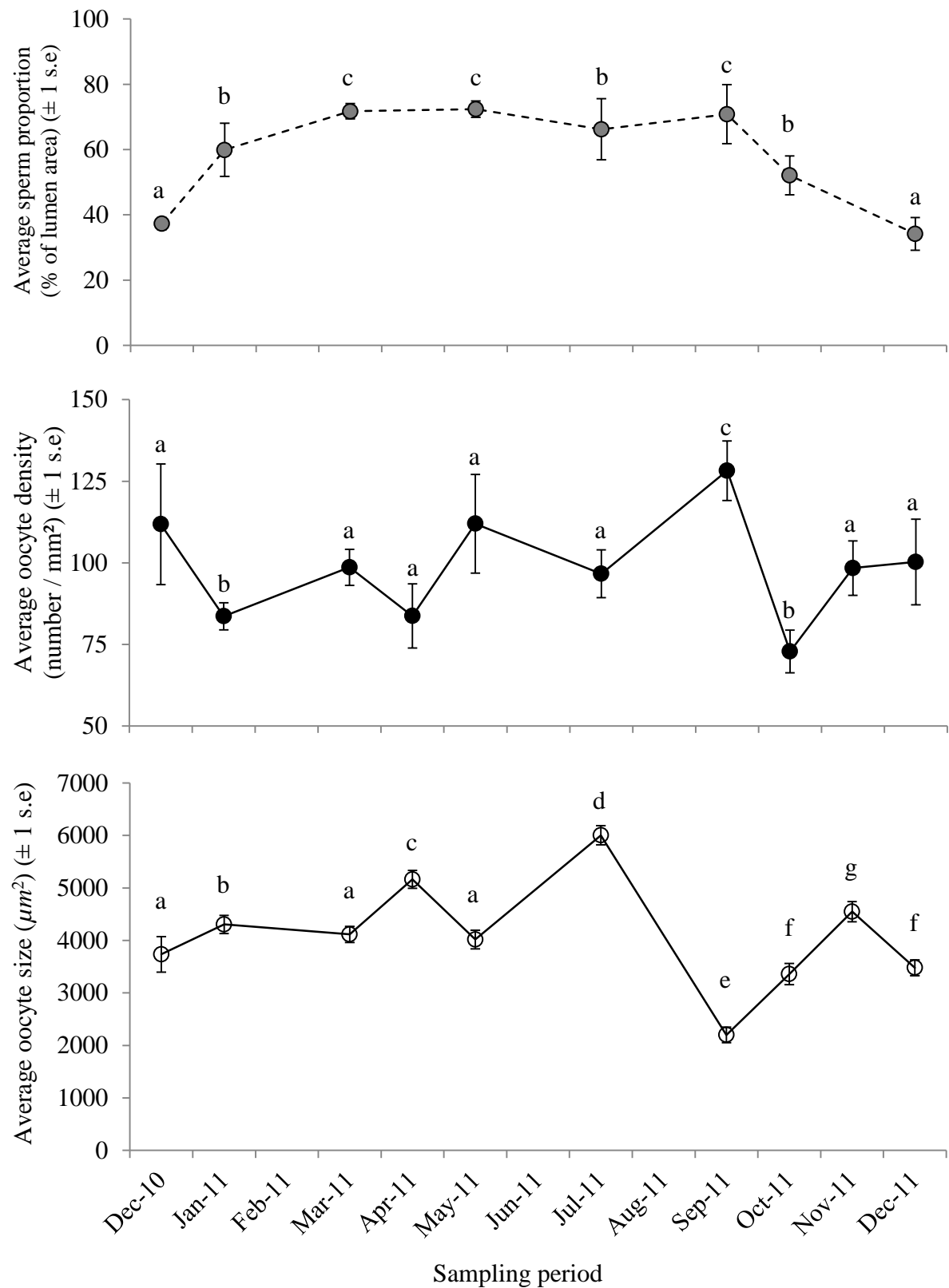
**Figure 4.2** Change in gonad index and shell index of *Liothyrella neozelanica* ( $n = 10$  for each month with the exception of December 2010 ( $n = 6$ ) and April 2011 ( $n = 8$ )). Gonad index is the ratio of gonad AFDM (mg) to overall body AFDM (mg). Shell index is the ratio of shell AFDM (mg) to overall body AFDM (mg) (Averages  $\pm 1$  s.e). Nb. Lower case letters denote significant differences among months for the gonad index ( $p < 0.05$ ).

### 4.3.2 Seasonal oocyte density

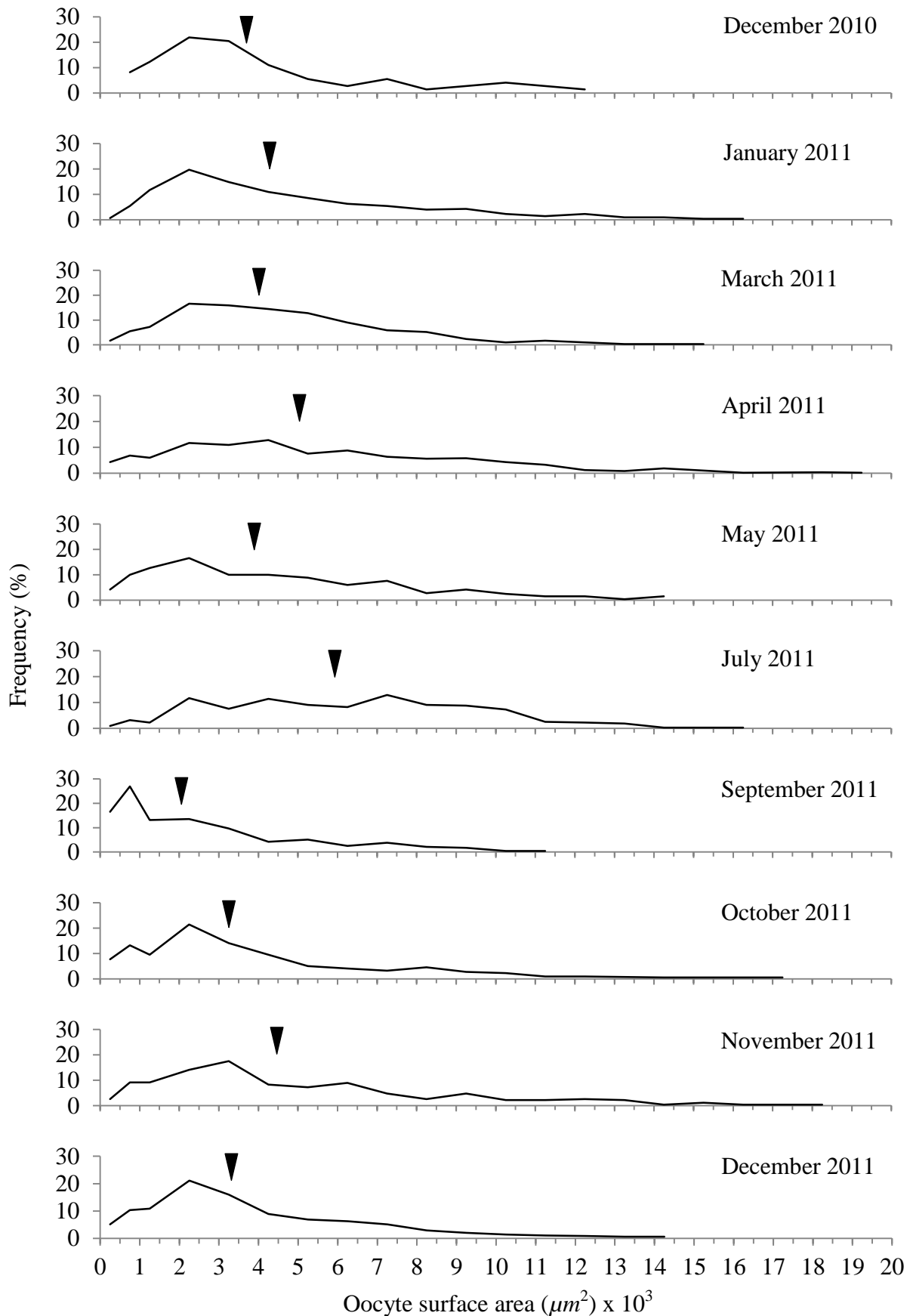
Average oocyte density was significantly different among months throughout the sampling period ( $F = 2.64$ ;  $df = 9, 64$ ;  $p = 0.013$ ) (Fig. 4.3). Average oocyte density in January 2011 (83.6 oocytes /  $\text{mm}^2$ ), September 2011 (128 oocytes /  $\text{mm}^2$ ) and October 2011 (73 oocytes /  $\text{mm}^2$ ) were significantly different from remaining sampled months (Fig. 4.3). Average oocyte density appeared relatively stable throughout December 2010 to July 2011 ranging between 84 and 112 oocytes /  $\text{mm}^2$ , with the exception of January 2011 (83.6 oocytes /  $\text{mm}^2$ ) which was significantly lower (Fig. 4.3). The average oocyte density increased 25% from July 2011 (97 oocytes /  $\text{mm}^2$ ) to reach a maximum of 128 oocytes /  $\text{mm}^2$  in September 2011. Average oocyte density decreased from September 2011 to reach a low of 73 oocytes /  $\text{mm}^2$ , before increasing and remaining steady throughout November 2011 (98 oocytes /  $\text{mm}^2$ ) and December 2011 (100 oocytes /  $\text{mm}^2$ ) (Fig. 4.3).

### 4.3.3 Seasonal oocyte size

Average oocyte size varied significantly among the sampled months ( $F = 30.95$ ;  $df = 9, 3015$ ;  $p < 0.0001$ ) (Fig. 4.3). Average oocyte size was relatively stable between December 2010 and May 2011 ranging between  $3735 \mu\text{m}^2$  (December 2011) and  $4114 \mu\text{m}^2$  (March 2011), with the exceptions of January 2011 ( $4306 \mu\text{m}^2$ ) and April 2011 ( $5164 \mu\text{m}^2$ ) which were significantly higher (Fig. 4.3). The size of oocytes between December 2011 and January 2011 were predominately between  $3500$  and  $4500 \mu\text{m}^2$  (Fig. 4.4). Higher numbers of oocytes ranging between  $5000 \mu\text{m}^2$  and  $10,000 \mu\text{m}^2$  began to appear over March 2011, April 2011 and May 2011, although large amounts of small oocytes ( $0 - 5000 \mu\text{m}^2$ ) were still present (Fig. 4.4). The size of oocytes in July 2011 predominately occurred between  $3000 \mu\text{m}^2$  and  $11,000 \mu\text{m}^2$  with an average of  $6003 \mu\text{m}^2$ , which was significantly higher than the averages of other sampled months (Fig 4.3 and 4.4). The average oocyte size in September 2011 decreased 3-fold from July 2011 to reach a low of  $2199 \mu\text{m}^2$ , which was reflected in high numbers of small oocytes ( $0 - 3000 \mu\text{m}^2$ ) (Fig 4.3 and 4.4). Average oocyte size increased steadily over October 2011 ( $3359 \mu\text{m}^2$ ) and November 2011 ( $4550 \mu\text{m}^2$ ) before significantly declining to  $3480 \mu\text{m}^2$  in December 2011 (Fig. 4.3). Oocyte size distribution between October 2011 and December 2011 appeared similar and reflected similar distributions observed in January 2011 and March 2011 (Fig. 4.3 and 4.4).



**Figure 4.3** Seasonal average sperm proportion (●) ( $n$ : Dec 10 = 3, Jan 11 = 4, Mar 11 = 4, May 11 = 4, Jul 11 = 2, Sep 11 = 4, Oct 11 = 3, Dec 11 = 5), average oocyte density (●) ( $n$ : Dec 10 = 3, Jan 11 = 10, Mar 11 = 4, Apr 11 = 5, May 11 = 6, Jul 11 = 5, Sep 11 = 7, Oct 11 = 6, Nov 11 = 13, Dec 11 = 6) and average oocyte size (○) ( $n$ : Dec 10 = 73, Jan 11 = 350, Mar 11 = 290, Apr 11 = 487, May 11 = 331, Jul 11 = 317, Sep 11 = 237, Oct 11 = 220, Nov 11 = 361, Dec 11 = 350) of *Liothyrella neozelanica* (Averages  $\pm$  1 s.e). Nb. Lower case letters denote significant differences among months ( $p < 0.05$ ).



**Figure 4.4** Seasonal oocyte size ( $\mu\text{m}^2$ ) frequency distributions of *Liothyrella neozelanica*. Black arrows represent seasonal oocyte size averages.

#### 4.3.4 Seasonal sperm proportion

Average sperm proportion varied significantly throughout the sampling period ( $F = 7.03$ ;  $df = 7, 28$ ;  $p = 0.0002$ ) (Fig. 4.3). Sperm proportion steadily increased 1.9 times from December 2010 (37.3 %) to March 2011 (71.7%), before remaining steady until September 2011 (70.8%), with the exception of July 2011 (66.2%) which was significantly lower (Fig. 4.3). Sperm proportion decreased from September 2011 (70.8%) over October 2011 (52.1%), reaching a low of 34.2% in December 2011 (Fig. 4.3).

#### 4.3.5 Oogenesis and spermatogenesis

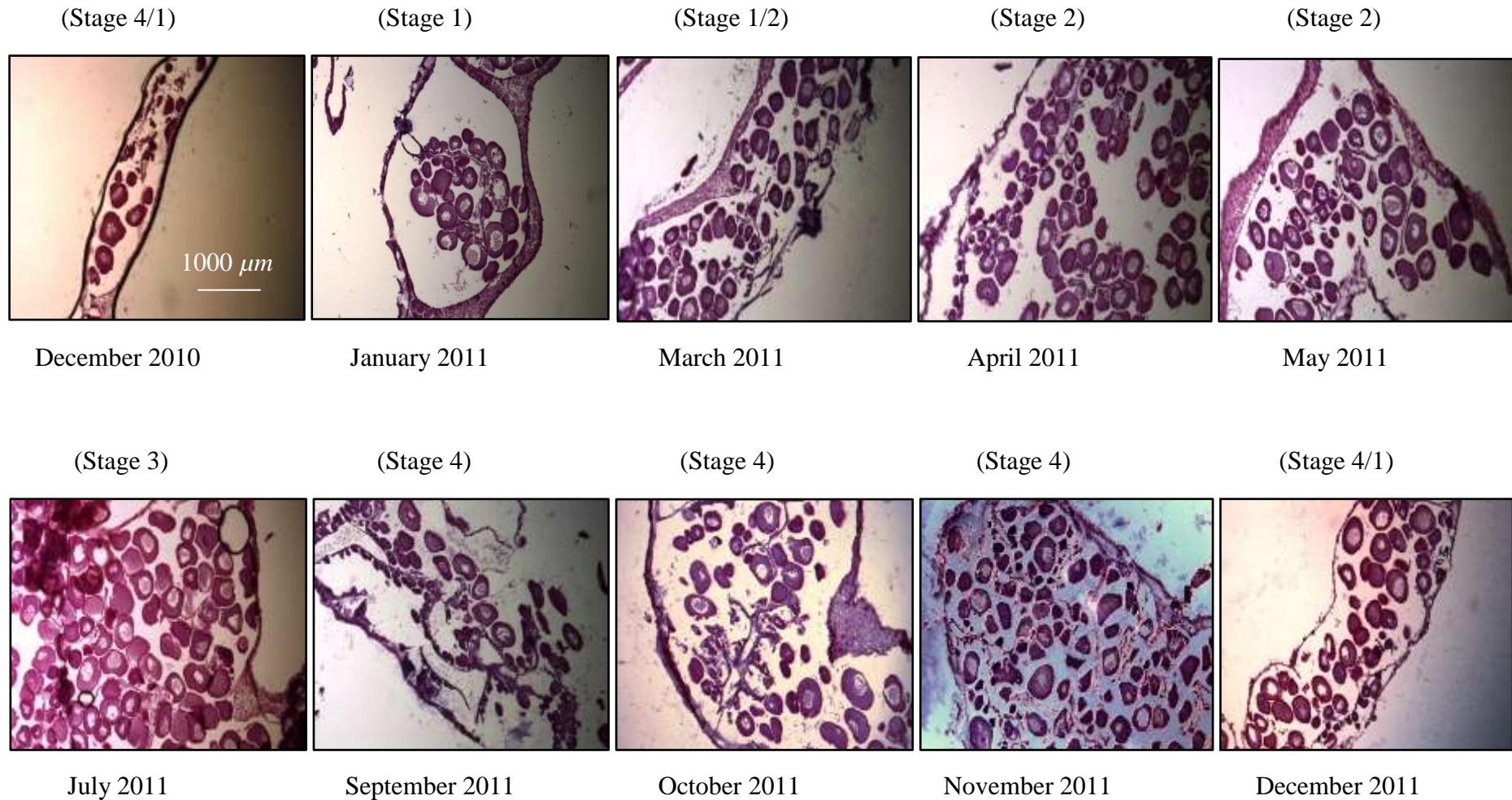
##### 4.3.5 (a) *Liothyrella neozelanica* oogenesis

Female histological observations throughout the sampling period are shown in Figure 4.4. Ovaries observed throughout December 2010 and January 2011 contained small undeveloped oocytes with no nucleus (Stage 1) (Fig. 4.5 and 4.6). Large, nucleated, oocytes attached near the ends of lamella began appearing between March 2011 and May 2011 (Stage 2) (Fig. 4.5 and 4.7). Large numbers of mature oocytes occupied the majority of lumen space in July 2011 (Stage 3) (Fig. 4.5 and 4.8). Ovaries observed throughout September 2011 and October 2011 held small numbers of mature oocytes and showed signs of necrotic tissue / lamella degradation (Stage 4) (Fig. 4.5 and 4.9). Ovaries observed in November 2011, contained necrotic oocytes (Stage 4) (Fig. 4.5). Ovaries observed in December 2011, appeared consistent with ovaries observed previously in December 2010 and January 2011, containing both mature and undeveloped oocytes, and holding remnant necrotic oocytes (Stage 1) (Fig. 4.5).

##### 4.3.5 (b) *Liothyrella neozelanica* spermatogenesis

Male *L. neozelanica* histological observations are shown in Figure 4.10. The area of lumen occupied by spermatozoa appeared to steadily increase across December 2010 to May 2011. Spermatozoa appeared to occur in low densities during July 2011 (Fig. 4.10). The amount of spermatozoa throughout September 2011 to November 2011 appeared to occur in high quantities (Fig. 4.10). The proportion of spermatozoa within the lumen in December 2011 appeared relatively low, with a similar appearance to the proportion observed in December 2010 (Fig. 4.10).

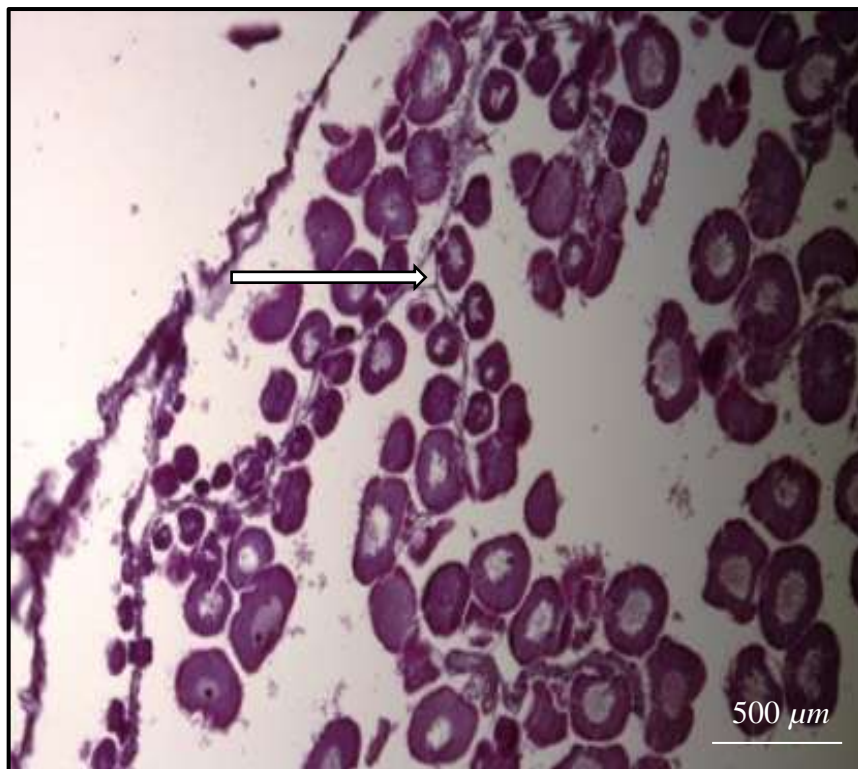




**Figure 4.5** Seasonal histological observations of female *Liothyrella neozelanica* gonads throughout the sampling period. Nb. All photos taken at 4 times magnification (Scale bar in December 2010 represents scale for all months).

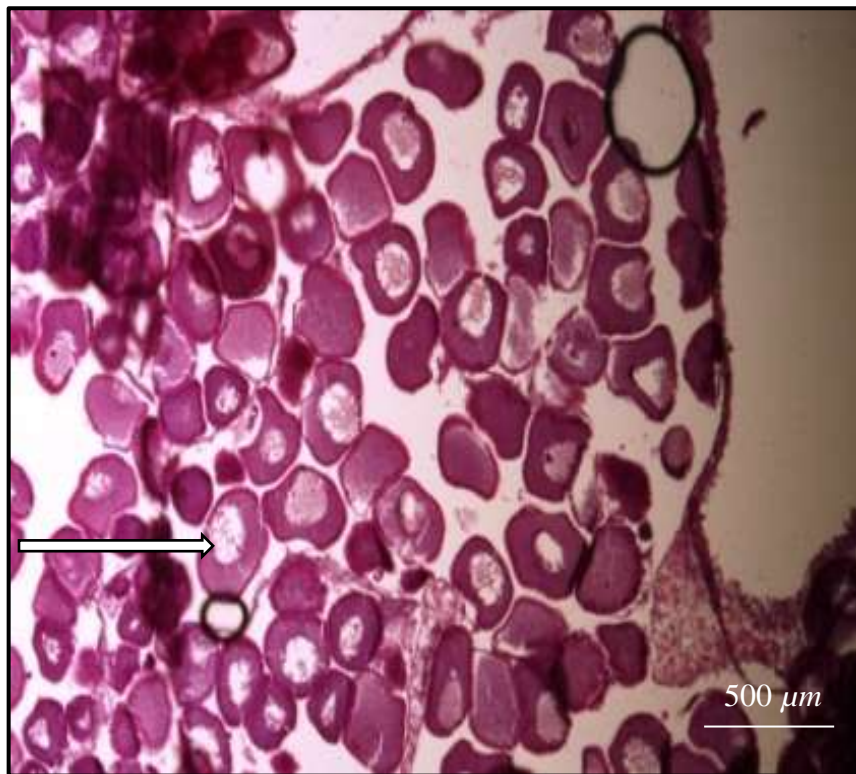


**Figure 4.6** *Liothyrella neozelanica* ovary showing stage 1 oogenesis in January 2011 (white arrow represents undeveloped oocytes).

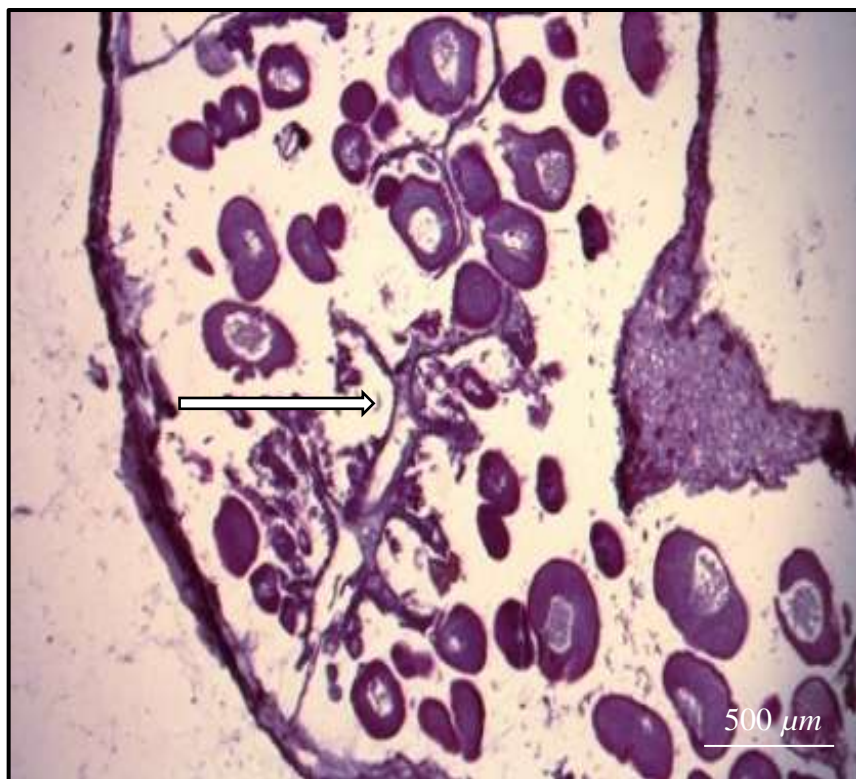


**Figure 4.7** *Liothyrella neozelanica* ovary showing stage 2 oogenesis in April 2011 (white arrow represents developing oocytes).

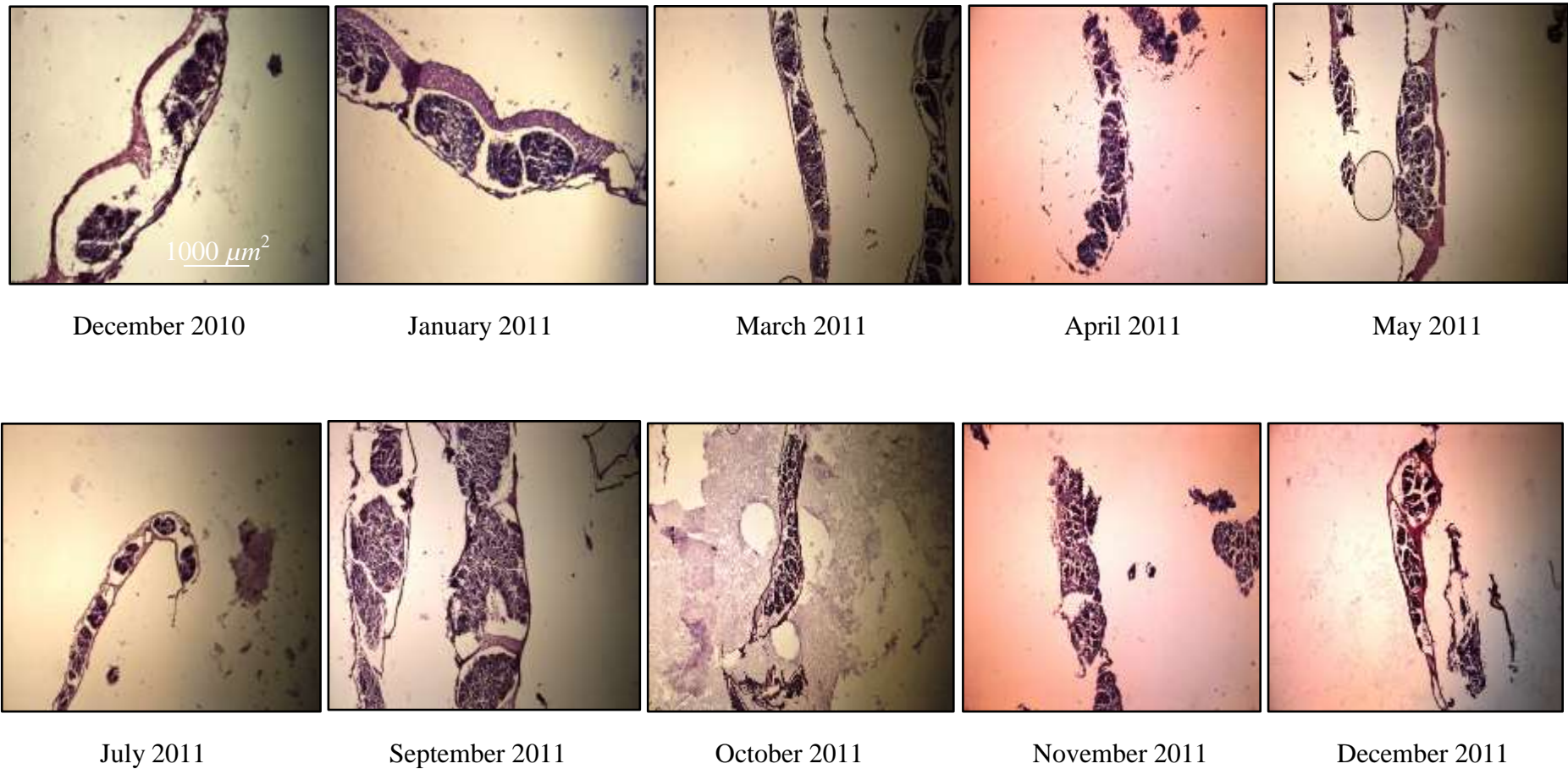




**Figure 4.8** *Liothyrella neozelanica* ovary showing stage 3 oogenesis in July 2011 (white arrow represents mature oocytes).



**Figure 4.9** *Liothyrella neozelanica* ovary showing stage 4 oogenesis in October 2011 (white arrow represents degrading lamella).



**Figure 4.10** Seasonal histological observations of male *Liothyrella neozelanica* gonads throughout the sampling period. Nb. All photos taken at 4 times magnification (Scale bar in December 2010 represents scale for all months).

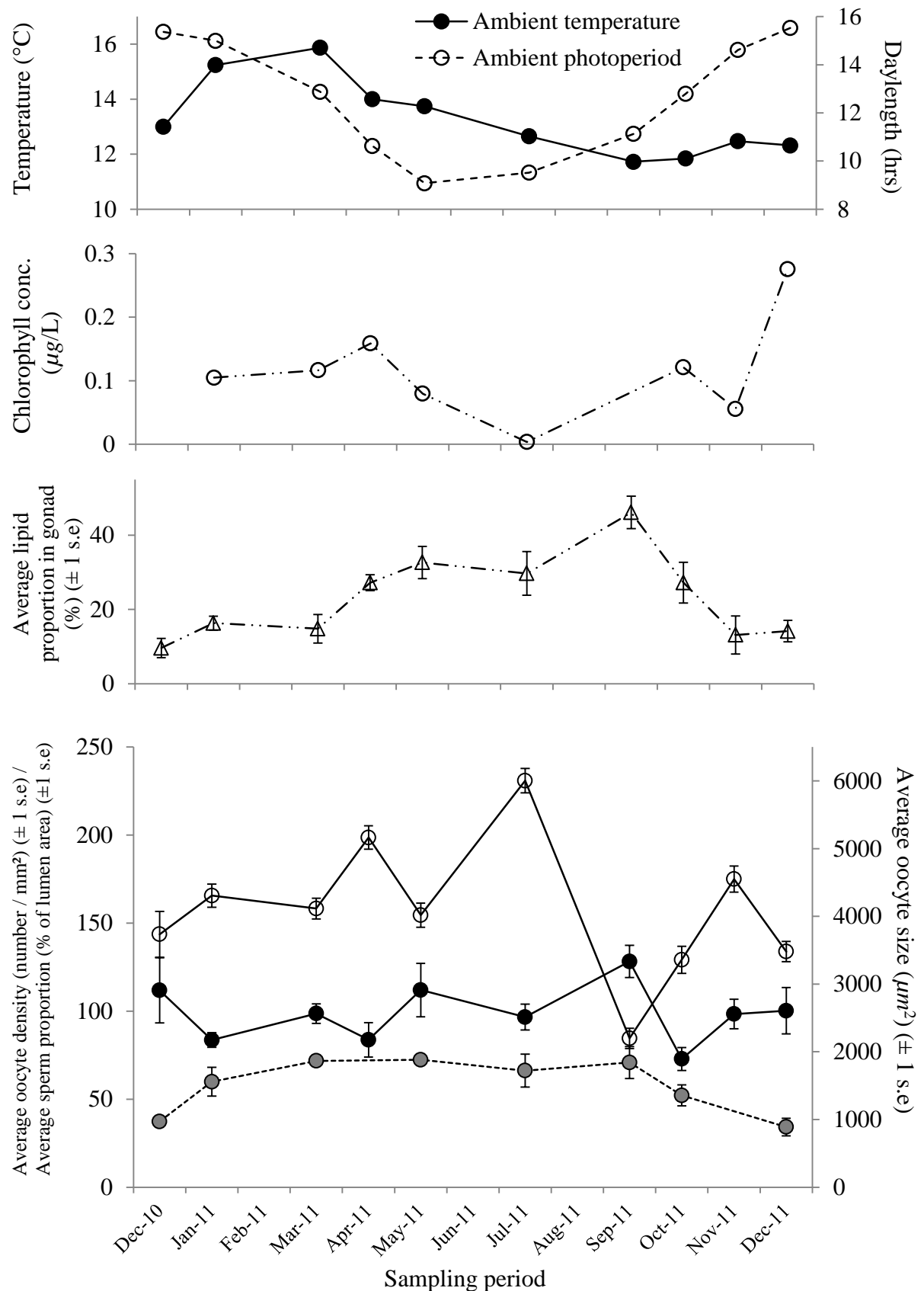
### 4.3.6 Seasonal factors and reproduction

#### 4.3.6 (a) Average oocyte density

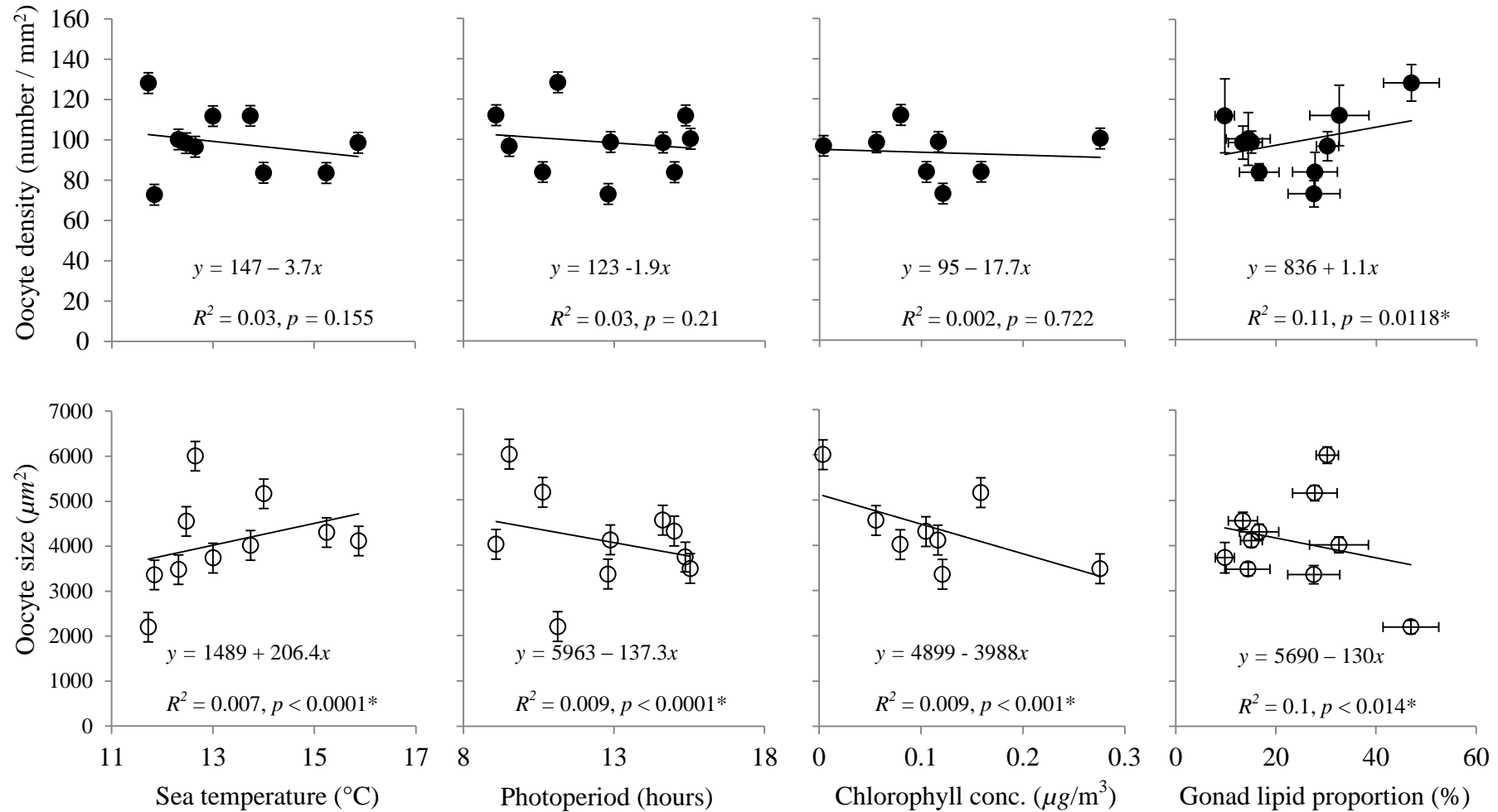
Average oocyte density followed a separate trend from those shown by ambient sea temperature, photoperiod and chlorophyll concentration throughout the sampling period (Fig. 4.11). Linear regressions revealed sea temperature ( $R^2 = 0.03$ ;  $F = 2.07$ ;  $df = 64$ ;  $p = 0.155$ ), photoperiod ( $R^2 = 0.03$ ;  $F = 1.63$ ;  $df = 64$ ;  $p = 0.21$ ) and chlorophyll concentration ( $R^2 = 0.002$ ;  $F = 0.13$ ;  $df = 54$ ,  $p = 0.722$ ) were not significantly related to oocyte density (Fig. 4.12). Oocyte density showed a similar trend to gonad lipid proportions throughout the sampling period, increasing between July 2011 and September 2011, before decreasing in October 2011 (Fig. 4.11). A linear regression between oocyte density and seasonal gonad lipid proportions showed a significant positive relationship ( $R^2 = 0.11$ ;  $F = 6.8$ ;  $df = 58$ ;  $p = 0.0118$ ) (Fig. 4.12).

#### 4.3.6 (b) Average oocyte size

Average oocyte size appeared to increase with decreasing sea temperature, however declined to a low of  $2198.8 \mu m^2$  in September 2011 as ambient sea temperature reached a minimum of  $11.7^\circ C$  (Fig. 4.11). A linear regression produced a significant positive relationship between sea temperature and average oocyte size ( $R^2 = 0.07$ ;  $F = 20.4$ ;  $df = 3015$ ;  $p < 0.0001$ ) (Fig. 4.12). Average oocyte size exhibited an opposite trend to those shown by ambient photoperiod and chlorophyll concentration and linear regressions revealed ambient photoperiod ( $R^2 = 0.009$ ;  $F = 28.3$ ;  $df = 3015$ ;  $p < 0.0001$ ) and ambient chlorophyll concentration ( $R^2 = 0.009$ ;  $F = 24.5$ ;  $df = 2705$ ,  $p < 0.001$ ) had a significant negative effect on average oocyte size (Fig. 4.12). Average oocyte size appeared to initially match trends shown by gonad lipid proportions however, average oocyte size decreased in September 2011 as lipid proportions reached a maximum of 46% (Fig. 4.11). A linear regression between average oocyte size and gonad lipid proportions produced a significant relationship ( $R^2 = 0.1$ ;  $F = 10.8$ ;  $df = 96$ ;  $p = 0.0014$ ) (Fig. 4.12).



**Figure 4.11** Seasonal ambient sea temperature (°C), photoperiod (hours), chlorophyll concentration (µg / L), gonad lipid proportion (%), average oocyte density (number / mm<sup>2</sup>) (●), average oocyte surface area (µm<sup>2</sup>) (○) and average sperm proportion (% of lumen area)(●) of *Liothyrella neozelanica* (Averages ± 1 s.e).

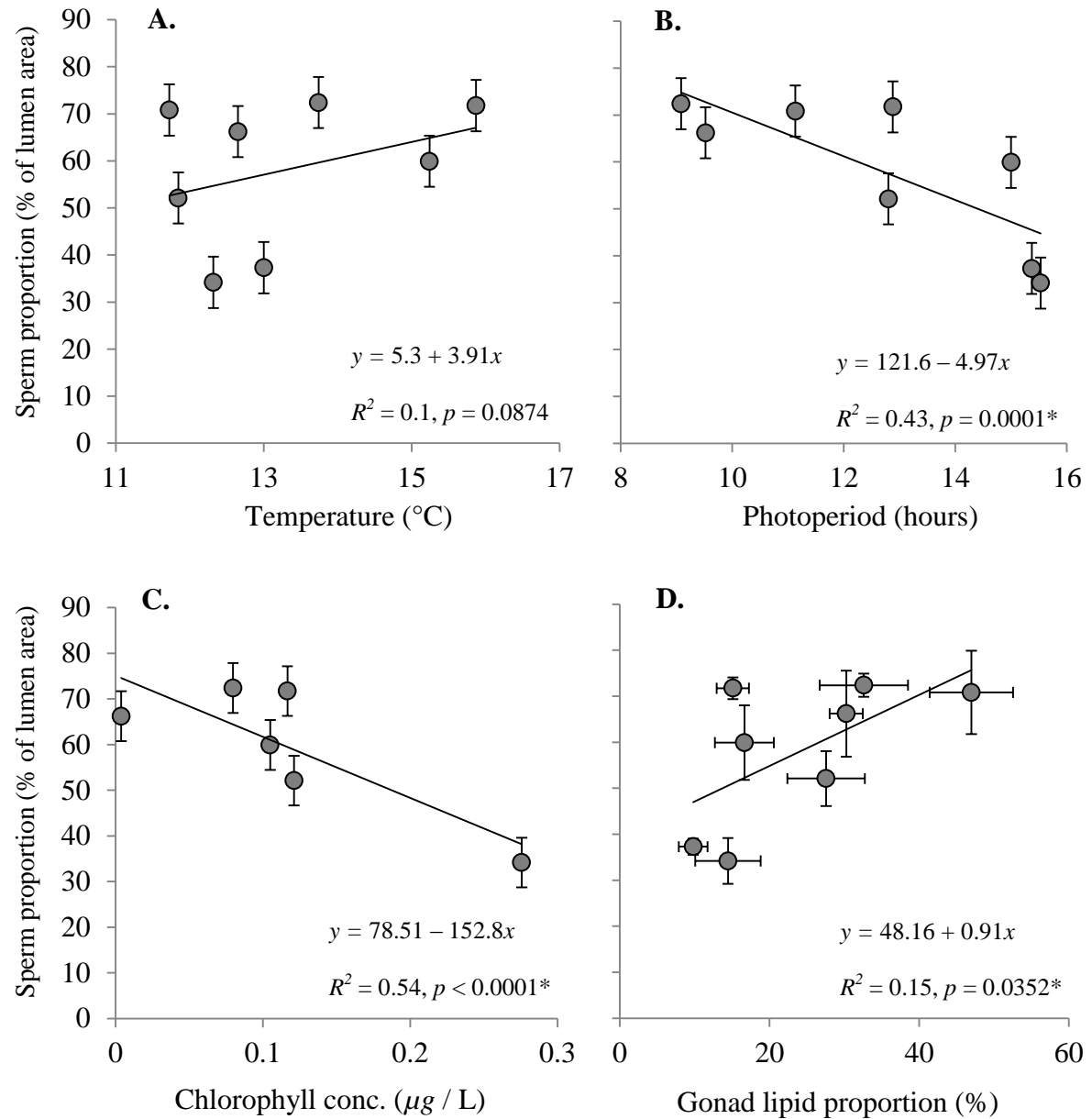


**Figure 4.12** Linear regressions between ambient sea temperature (°C), photoperiod (hours), chlorophyll concentration (µg / L), gonad lipid proportion (%), average oocyte density (number / mm<sup>2</sup>) ( $\pm 1$  s.e) and average oocyte size (µm<sup>2</sup>) ( $\pm 1$  s.e) (Oocyte density: temperature ( $n = 65$ ), photoperiod ( $n = 65$ ), chlorophyll ( $n = 55$ ), gonad lipid proportion ( $n = 59$ ); Oocyte size: temperature ( $n = 3016$ ), photoperiod ( $n = 3016$ ), chlorophyll ( $n = 2706$ ), gonad lipid proportion ( $n = 97$ ) (\* denotes significance).

**4.3.6 (b) Average sperm proportion**

Average sperm proportion showed no similarity in trend to ambient sea temperature throughout the sampling period and a linear regression between the two produced an insignificant relationship ( $R^2 = 0.1$ ;  $F = 3.15$ ;  $df = 28$ ;  $p = 0.09$ ) (Fig. 4.13). Average sperm proportion appeared to follow an opposite trend to those shown by ambient photoperiod and chlorophyll concentration, increasing in winter and decreasing in summer (Fig. 4.11). Linear regressions showed ambient photoperiod ( $R^2 = 0.43$ ;  $F = 20.36$ ;  $df = 28$ ;  $p = 0.0001$ ) and chlorophyll concentration ( $R^2 = 0.5$ ;  $F = 23.8$ ;  $df = 21$ ;  $p < 0.0001$ ) had significant negative effects on the proportion of sperm within the lumen (Fig. 4.13). Average sperm proportion appeared to follow a similar trend shown by gonad lipid proportions; increasing over winter and decreasing over summer (Fig. 4.11). A linear regression showed gonad lipid proportion ( $R^2 = 0.15$ ;  $F = 3.15$ ;  $df = 28$ ;  $p = 0.04$ ) to have a significant positive relationship with sperm density (Fig. 4.13).





**Figure 4.13** Linear regressions between ambient sea temperature (°C) (A), photoperiod (hours) (B), chlorophyll concentration ( $\mu\text{g} / \text{L}$ ) (C), gonad lipid proportion (%) (D) and average sperm proportion (% of lumen area) (Averages  $\pm$  1 s.e) (Sperm proportion: temperature ( $n = 29$ ), photoperiod ( $n = 29$ ), chlorophyll concentration ( $n = 22$ ), gonad lipid proportion ( $n = 29$ )) (\* represents significance).

## 4.4 Discussion

### 4.4.1 Reproductive development and spawning

The gonad index of *Liothyrella neozelanica* increased throughout December 2010 to May 2011 by 9.8 %, before steadily declining and remaining steady throughout July 2011 to December 2011. This suggests that *L. neozelanica* may have undergone a spawning event sometime between May 2011 and July 2011. However this finding is inconsistent with those trends observed from the gametogenic examinations.

Average oocyte density and average oocyte size displayed an inverse relationship throughout the study period, where decreasing oocyte density coincided with increasing oocyte size and vice versa. This relationship between oocyte density and size is likely due to *Liothyrella neozelanica* larvae being lecithotrophic; therefore oocytes are relatively large as a result of yolk reserves. This has been shown to occur in other marine invertebrates with lecithotrophic larvae, including the echinoid brittle star *Ophiopholis aculeate*, where large, mature oocytes take up greater amounts of lumen space and therefore reduce oocyte density (Doyle *et al.* 2012).

Oocyte density remained relatively stable between December 2010 (112 oocytes / mm<sup>2</sup>) and July 2011 (96 oocytes / mm<sup>2</sup>), however increased to a maximum of 128 oocytes / mm<sup>2</sup> in September 2011 as average oocyte size decreased 3- fold from July 2011 to a minimum of 2199  $\mu\text{m}^2$ . Significant reduction in average oocyte size has been linked to spawning episodes in other marine invertebrates including the echinoderm *Ophiopholis aculeate* (Doyle *et al.* 2012), the mollusc *Mytilus edulis* (Mackie 1984) and the Antarctic brachiopod *Liothyrella uva* (Meidlinger *et al.* 1998). In this study, the greatest decrease in average oocyte size (63% change) occurred between July 2011 (6003  $\mu\text{m}^2$ ) and September 2011 (2199  $\mu\text{m}^2$ ). Furthermore, the size distribution of oocytes in July 2011 ranged predominately between 3000 and 11,000  $\mu\text{m}^2$  before declining in September 2011 to lie predominately between 0 and 3000  $\mu\text{m}^2$ . Moreover, large numbers of mature oocytes were present in the lumen in July 2011, but were lower in number in September 2011. At the same time there was evidence of lamella degradation and necrotic oocytes throughout October 2011 and November 2011. Lastly, a female *L. neozelanica* individual freshly collected from Tricky Cove (< 1 hour) showed signs of possible brooding in September 2011 (Appendix 3.1). These collaborative findings all

suggest that *L. neozelanica* underwent a spawning event between the end of winter (July 2011) and the start of spring (September 2011). This finding agrees with Lee *et al.* (2010) who stated that based on the size of juveniles on settling plates that *L. neozelanica* may spawn during spring.

A possible explanation for the trends shown in the gonad index, where spawning appears to take place between May and July, may be a result of the proportions of AFDM found in the shell of *L. neozelanica* throughout the sampling period. The shell of *L. neozelanica* represents the highest proportion of AFDM in the overall body AFDM weight of *L. neozelanica*. As a result it appeared to influence the gonad index by either decreasing the proportion of gonad when shell weight was high, or increasing the proportion of gonad when shell weight decreased. Therefore it is uncertain whether the gonad index reflects gonadal development or whether it reflects change in shell tissue weight throughout the sampling period. Furthermore, Lurman *et al.* (2010) found that internal tissue, in particular the adductor muscle of *L. neozelanica*, increased in size by 26% over the winter to metabolically counteract cooling sea temperature by increasing myocyte numbers within the tissue. As a result increased tissue throughout winter may have influenced proportions of gonad in respect to overall body weight. Due to the lack of clarity concerning the AFDM gonad index of *L. neozelanica*, trends should be observed with caution and further research is needed to clarify whether the reproductive development of *L. neozelanica* can be effectively traced using AFDM gonad to body proportions.

Interestingly the proposed spawning time of *L. neozelanica* females between late July and early September does not agree with previously published findings by Tortell (1981) and Chuang (1994) who observed spawning by *L. neozelanica* females within the laboratory during February and March. Tortell (1981) observed that 3 out of 8 female *L. neozelanica* were spent and one female held mature oocytes in February. However, the female *L. neozelanica* observed by Tortell (1981) showed signs of dehydration and autolysis as they had been held out of water for greater than 24 hours and as a result, it was noted that the findings were tentative and may not be truly representative of natural populations (Tortell 1981). Chuang (1994) observed natural spawning in 9 of 87 individual female *L. neozelanica* (10%) within the laboratory and found the majority of females contained mature oocytes throughout February and March. Within this study, mature oocytes were found within the lumen of

female *L. neozelanica* throughout January 2011 and March 2011, albeit in small numbers compared to those exhibited in July 2011. It has been noted by James *et al.* (1992) and Chuang (1990) that slight elevations in water temperature and physical agitation can trigger spawning in Rhynchonelliform brachiopods, which was also personally observed within this study by several *L. neozelanica* individuals that were shaken. Although it is unknown whether *L. neozelanica* individuals within the study of Chuang (1994) were kept in ambient conditions, it is possible that the 10% of female individuals that spawned within the laboratory may have been triggered by stress, as there was no clear evidence of female *L. neozelanica* spawning throughout February and March under ambient conditions within this study.

It must be noted however, that the range of observations of *L. neozelanica* spawning, together with the presence of large mature oocytes within female *L. neozelanica* throughout the entire sampling period, may indicate *L. neozelanica* is capable of spawning multiple times throughout an annual period. Furthermore, a population of temperate *Terebratulina retusa* brachiopods has been shown to spawn multiple times throughout spring, summer and autumn (James *et al.* 1991b), and the New Zealand brachiopod *Calloria inconspicua* has been shown to spawn in both April and August (Doherty 1979). Therefore, although *L. neozelanica* appears to only spawn in spring within this study, further research over multiple annual periods is needed to clarify whether *L. neozelanica* exhibits multiple spawning events.

Average oocyte size decreased by 43% between September 2011 (128 oocytes / mm<sup>2</sup>) and October 2011 (73 oocytes / mm<sup>2</sup>) as average oocyte size steadily increased. The reduction in average oocyte density may be explained by the high number of necrotic oocytes observed in October 2011, suggesting that unspent oocytes may have been being re-absorbed. This re-absorption of oocytes (oosorption) has also been observed and well documented in the Rhynchonelliform brachiopod *Terebratulina retusa* (James *et al.* 1991a, c).

#### 4.4.2 Seasonal spermatogenesis

Sperm proportions within the lumen of male *L. neozelanica* increased between December 2010 (37% of lumen area) and March 2011 (72% of lumen area), before holding relatively steady until October 2011. Sperm proportion decreased from September 2011 (71% of lumen

area) to December 2011 reaching levels similar to those observed in December 2010 (32% of lumen area). Female *L. neozelanica* are known to brood their larvae (Chuang 1994) and Long and Stricker (1991) suggested that brooding by female brachiopods is an adaptation whereby females hold mature oocytes within their mantle cavity and use feeding currents generated by the lophophore to draw in sperm to initiate fertilisation. The drop in average oocyte size observed in female *L. neozelanica* in September 2011 may indicate the release of mature oocytes into the mantle cavity, whereby sperm is released by male *L. neozelanica* over September 2011 and October 2011 to enable fertilisation. This explanation would again agree with findings by Lee *et al.* (2010) who showed evidence of a possible spawning event occurring in spring, based on larval recruitment research.

Male *L. neozelanica* sperm proportions observed between December 2010 to March 2011 steadily increased and showed no sign of decreasing between January 2011 and March 2011, which is the previously proposed spawning time of *L. neozelanica* suggested by Tortell (1981) and Chuang (1994). Furthermore, sperm proportion decreased from September 2011 (71% of lumen area) to reach a minimum recorded amount of 34% of lumen area in December 2011, suggesting spawning had occurred over this time. These findings again contradict previously suggested spawning times (February to March) and indicate that spawning most likely occurs between the end of July 2011 and September 2011 as spring begins.

#### **4.4.3 Factors influencing reproductive development**

##### **4.4.3 (a) Oocyte density**

Oocyte density was found to have no significant relationship with ambient sea temperature, photoperiod and ambient chlorophyll concentration, suggesting the production of new cohorts is independent of surrounding environmental conditions. This independent production of new cohorts may be an adaptation by *L. neozelanica*, enabling it to undergo small spawning events throughout the year when conditions are favourable. This is further supported by the presence of mature oocytes within *L. neozelanica* throughout the majority of the year and previous observations of spawning taking place during February and March in the laboratory (Tortell 1981; Chuang 1994). Further research over multiple years is needed to clarify this.

Oocyte density was found to be positively correlated to gonadal lipid proportion, with higher levels of lipid occurring within *L. neozelanica* gonads as oocyte density increased. This is consistent with previous findings involving Rhynchonelliform brachiopods, whereby lipid is often a major component of yolk within oocytes (Long 1964; James *et al.* 1992). Therefore it is expected that as oocyte densities increase, the proportion of lipid within the gonad increases. This is further supported by lipid levels decreasing 1.7 times, between September 2011 and October 2011, coinciding with a 43% reduction in oocyte density.

#### 4.4.3 (b) Oocyte size

A linear regression showed that average oocyte size was significantly positively related to ambient sea temperature throughout the sampling period, however it should be noted that the fit of the regression was extremely weak ( $R^2 = 0.007$ ), suggesting high variability within the model. Interestingly, the highest average oocyte size in July 2011 ( $6003 \mu m^2$ ) occurred when ambient sea temperature was  $12.7^\circ C$  and was followed by the lowest average oocyte size recorded in September 2011 ( $2199 \mu m^2$ ), as ambient sea temperature dropped below  $12^\circ C$  to reach a recorded minimum of  $11.7^\circ C$ . Bivalves have been shown to link their spawning events to temperature in many different ways (Mackie 1984). The temperate bivalves *Mytilus edulis* and *Mya arenaria* link their spawning to temperatures that exceed a temperature threshold of 10 to  $12^\circ C$ , whereas the horse clam *Tresus capax* initiates gametogenesis when the season minimum temperature is reached (Mackie 1984). *Liothyrella neozelanica* shows evidence within this study of coinciding spawning (reduction in average oocyte size) with the seasonal minimum temperature recorded, similar to the horse clam *T. capax* (Quayle and Bourne 1972).

The average oocyte size of *L. neozelanica* was found to be negatively correlated to ambient photoperiod, with greater average oocyte sizes occurring when photoperiod was low. Furthermore, average oocyte size was found to be negatively correlated to ambient chlorophyll concentration, with greater average oocyte sizes occurring when chlorophyll concentrations were low. The average oocyte size, ambient photoperiod and ambient chlorophyll concentration regressions, suggest that oocyte development occurs over winter as photoperiod and chlorophyll concentrations are low. Interestingly average oocyte size significantly decreased in September 2011 from July 2011 as ambient photoperiod and

ambient chlorophyll concentration began to increase, suggesting that increasing ambient photoperiod and chlorophyll concentration may act as environmental cues, triggering spawning within *L. neozelanica*.

Spawning events in spring coinciding with increased photoperiod and phytoplankton concentration, has been observed in the temperate brachiopod *Terebratulina retusa* (James *et al.* 1991b) and the Antarctic brachiopod *Liothyrella uva* (Meidlinger *et al.* 1998). Furthermore, it has been suggested by Lawrence and Soame (2004) that photoperiod is the major reliable environmental cue used by the majority of marine invertebrates to synchronise spawning events, as temperature can vary greatly inter-seasonally and inter-annually, possibly causing spawning at irregular times. Based on these findings it is reasonable to suggest that in *L. neozelanica*, spawning coincides with the onset of spring as photoperiod and food availability increase, which may act to enable greater larval survival success.

Average oocyte size was found to be significantly negatively correlated to gonad lipid proportions throughout the sampling period. This finding is contradictory to previous findings involving brachiopod development, which state large oocytes hold greater amounts of lipid compared to comparatively smaller oocytes (James *et al.* 1992). An explanation for the observed negative trend may be explained by high proportions of gonad lipid coinciding with an increase in average oocyte density in September 2011. Despite average oocyte size decreasing in September 2011, average oocyte density reached a maximum of 128 oocytes / mm<sup>2</sup>, as large numbers of small oocytes appeared in the ovaries of *L. neozelanica*. As a result, the high density of small oocytes appeared to maintain high gonad lipid proportions, despite a decrease in average oocyte size.

#### **4.4.3 (c) Sperm proportion**

Average sperm proportion within *L. neozelanica* was not significantly related to ambient sea temperature throughout the sampling period, suggesting spermatogenesis may develop independently of temperature. Linear regressions between average sperm proportion, ambient photoperiod and ambient chlorophyll both produced significant negative relationships, where high proportions of sperm occurred when photoperiod and chlorophyll concentrations were

low. These negative relationships are similar to those exhibited in the average oocyte size, photoperiod and chlorophyll concentration regressions, suggesting that reproductive development within male and female *L. neozelanica* may be synchronous. Lowest sperm proportions occurred in both December 2010 (37% of lumen area) and December 2011 (34% of lumen area) as ambient chlorophyll concentration ( $0.28 \mu\text{g} / \text{L}$ ) and ambient photoperiod (15.5 hours) reached their peaks. Furthermore sperm proportions steadily decreased from September 2011 to December 2011 as ambient chlorophyll concentration and ambient photoperiod increased, suggesting that spawning in male *L. neozelanica* may be triggered by increasing ambient photoperiod and chlorophyll concentration.

A linear regression between gonad lipid levels and average sperm proportion revealed a significant positive relationship suggesting that increasing sperm proportions cause an increase in gonadal lipid proportions. Energy reserves in brachiopod sperm is relatively undefined, however it has been suggested that sperm within the temperate *Terebratulina retusa* brachiopod contain phospholipids as energy reserves, as seen in sea urchins (Afzelius and Mohri 1966; James *et al.* 1992). Therefore it seems reasonable to suggest that *L. neozelanica* sperm accumulate reserves of phospholipid during development, reflected in the observed linear relationship between sperm proportion and gonad lipid proportion.

#### **4.4.4 Climate change implications**

Linear regressions between oocyte density, sperm proportion and ambient sea temperature showed no relationship suggesting that these processes develop independently from ambient sea temperature. Furthermore, the linear regression between oocyte size and ambient sea temperature contained a lot of variability suggesting again that ambient sea temperature may not be as important in *L. neozelanica* reproductive development as other environmental variables.

Within this study, clear negative relationships between photoperiod, chlorophyll concentration, average oocyte size and average sperm proportion were observed, suggesting that these environmental factors may be the main drivers in triggering reproductive development and spawning within *L. neozelanica*. These findings are also consistent with Lawrence and Soame



(2004) who suggest that photoperiod is likely the major environmental variable driving reproductive development in marine invertebrates. Due to these findings, a 2°C increase in ambient sea temperature resulting from climate change (IPCC 2007) may not directly affect the reproductive cycle of *L. neozelanica*, as reproduction appears to be independent of ambient sea temperature and more closely related to fluctuations in ambient photoperiod and ambient chlorophyll concentration. However, an increase in sea temperature may act to indirectly increase water column stratification, thereby reducing the amount of primary production reaching the euphotic zone (Harley *et al.* 2006). This may ultimately lead to an affect on *L. neozelanica* reproduction over the next century. Further research is needed to address this issue.

Interestingly, small increases in water temperature within the laboratory have been shown to cause spawning within some Rhynchonelliform brachiopods (Chuang 1990; James *et al.* 1992). This suggests a 2°C increase in ambient sea temperature may affect the reproductive development of some Rhynchonelliform brachiopod species in the future. However, *L. neozelanica* already experiences large inter-seasonal / inter-annual sea temperature fluctuations within its ambient environment. Therefore it seems reasonable to suggest that *L. neozelanica* may have cued its reproductive development to more stable environmental factors such as photoperiod and will therefore remain relatively reproductively unaffected in the future as sea temperatures rise by 2°C (IPCC 2007).

#### 4.4.5 Conclusion

Gametogenic development observations within this study have provided evidence suggesting *L. neozelanica* spawned between the end of winter (July 2011) and the start of spring (September 2011) during the December 2010 to December 2011 period. Spawning was evident by a strong decrease in average oocyte size and the appearance of high numbers of small oocytes. Furthermore, evidence of lamella degradation and necrotic tissue in the lumen of *L. neozelanica* throughout October 2011 and November 2011 supports a spawning event occurring between July 2011 and September 2011. A spawning event in spring as suggested in this study supports previous spawning evidence of *L. neozelanica*, based on a tile recruitment study (Lee *et al.* 2010). However, the presence of large, mature oocytes within the ovaries of *L. neozelanica* throughout the majority of the sampling period, possibly suggest

that *L. neozelanica* possesses the ability to undergo multiple spawning events throughout a year. Further reproductive research over a multiple annual period is needed to verify this issue.

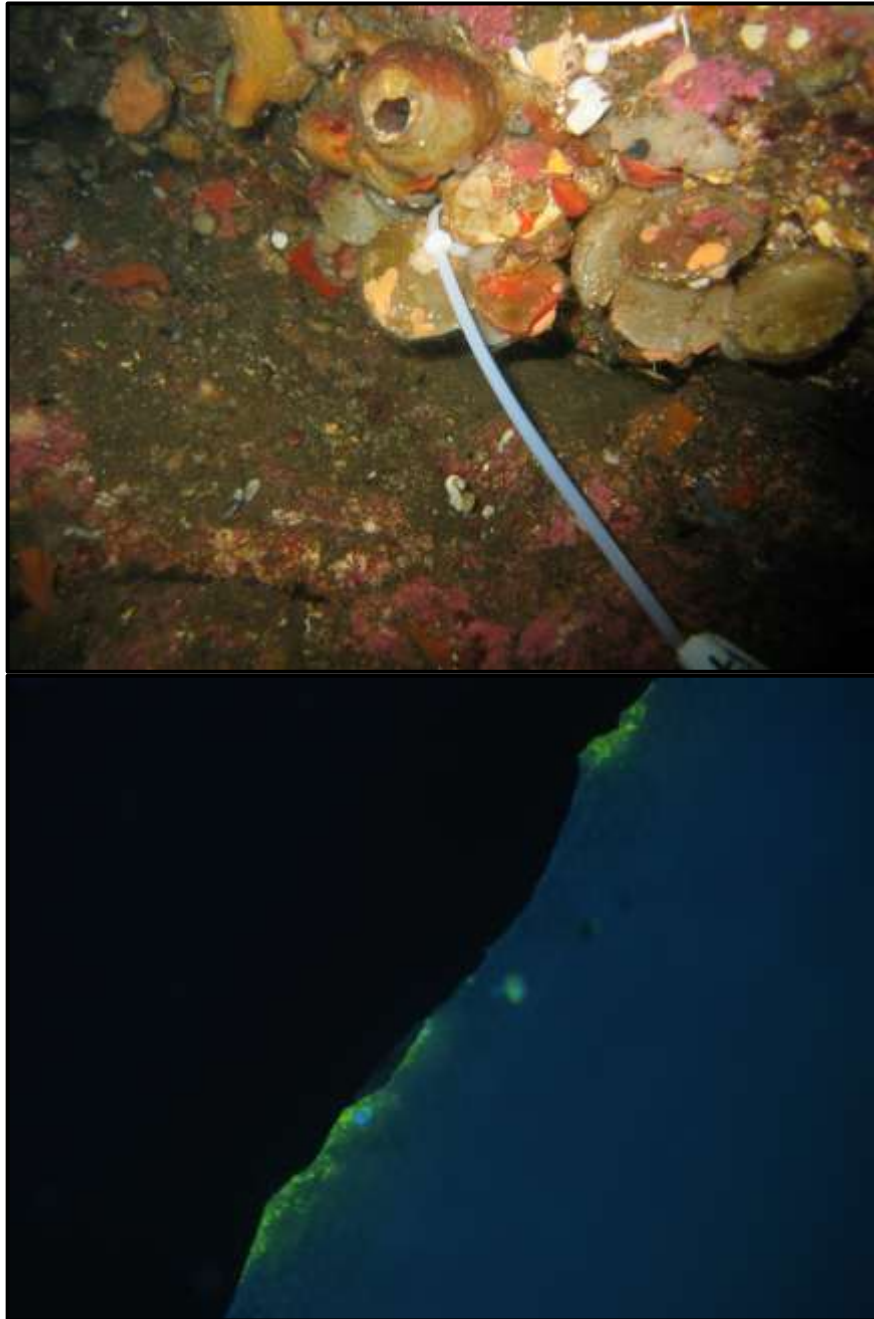
Photoperiod and ambient chlorophyll concentration were found to have significant negative relationships with oocyte size and sperm proportion, suggesting that they may act as environmental cues to trigger synchronous spawning of male and female *L. neozelanica* brachiopods at the beginning of spring. Gonad lipid proportions were found to be significantly positively related to average oocyte density and average sperm proportion, suggesting that lipid is the main energy reserve within the gametes of *L. neozelanica*; stored as granules within oocytes and as phospholipids within spermatozoa.

As *L. neozelanica* presently experiences large inter-seasonal / inter-annual ambient sea temperature fluctuations, it is likely that its reproductive development has been cued to more stable environmental factors such as photoperiod and chlorophyll concentration. As a result, it is suggested in this study that the reproductive cycle of *L. neozelanica* will be relatively unaffected in the future as sea temperatures rise by 2°C (IPCC 2007). However, further research is needed to clarify whether lower levels of primary productivity via increased stratification (Harley *et al.* 2006) may affect reproductive development in the future.



# CHAPTER 5

## ANNUAL GROWTH OF *LIOTHYRELLA NEOZELANICA*



Top: A manually tagged *Liothyrella neozelanica*. Bottom: A fluorescing tetracycline tag

**“You’ve got to do your own growing, no matter how tall your grandfather was”**

**(Irish proverb)**

## 5.1 Introduction

Growth of marine invertebrates has been well documented with examples from every phylum, particularly those phyla that are abundant and / or commercially important. As a result, phyla such as the marine molluscs, in particular bivalves (Kaehler and McQuaid 1999; Gosling 2003), clams (Cranfield and Michael 2001) and gastropods (Pirker and Schiel 1993) have received a lot of attention. In contrast, the growth of brachiopods is comparatively undefined as they are often patchily distributed and viewed as commercially unimportant (Richardson 1986). Despite this, understanding the growth of living brachiopods is necessary as they often serve as ecologically important constituents in areas where they occur and provide vital insight into understanding population dynamics within fossil records (Doherty 1979).

To date, the growth of five species of temperate Rhynchonelliform brachiopods have been quantified; *Terebratalia transversa* (Thayer 1977), *Calloria* (*Terebratella*) *inconspicua* (Doherty 1979), *Terebratulina retusa* (Curry 1982; Collins 1991) *Neothyris lenticularis* (Aldridge 1999) and *Terebratella sanguinea* (Ostrow 2007), along with three species of Antarctic Rhynchonelliform brachiopods; *Magellania fragilis* (Brey *et al.* 1995), *Liothyrella uva* (Peck *et al.* 1997) and *Neorhynchia strebli* (Barnes and Peck 1997). Three techniques were utilised in these studies to determine growth rates: 1) determining size-frequency distributions within a population (Thayer 1977; Doherty 1979; Collins 1991); 2) measuring annual growth rings (Doherty 1979; Brey *et al.* 1995; Aldridge 1999); 3) manually tagging animals and measuring growth over an annual period (Doherty 1979; Peck *et al.* 1997; Ostrow 2007). Of these techniques, tagging animals and measuring growth is the most reliable as size-frequency distributions rely heavily on samples reflecting actual size structures of populations and the measurement of annual growth rings depends entirely on whether visible rings are a true reflection of annual growth, which is often unknown (Gosling 2003).

Three out of the eight studies involving Rhynchonelliform brachiopods used individual tagging to investigate growth rates (Doherty 1979; Peck *et al.* 1997; Ostrow 2007). Doherty (1979) reported no growth in 14 tagged *C. inconspicua* individuals (> 18 mm width) over a 248 day period, whereas Ostrow (2007) described maximum growth ranging from 4 to 12 mm yr<sup>-1</sup> in *T. sanguinea* depending on the sampling site. However, it was concluded by Ostrow

(2007) that the results should be viewed cautiously as the sample size was small ( $n = 24$ ) and the variation substantial. Peck *et al.* (1997) observed *L. uva* to grow at a rate of  $1.6 - 2.3 \text{ mm yr}^{-1}$  in 5 mm long tagged individuals and  $0.96 - 1.44 \text{ mm yr}^{-1}$  in 20 mm long tagged individuals, which was found to be 15% to 50% lower than those reported for temperate brachiopods (Peck *et al.* 1997). However, the growth comparisons between temperate and Antarctic brachiopods examined by Peck *et al.* (1997) involved brachiopods from different genera and results that were obtained using different methodologies (Doherty 1979; Collins 1991).

Collectively the eight Rhynchonelliform brachiopod growth studies have provided vital insights into population dynamics, life-histories, evolution, seasonal energetics and the ecological importance of Rhynchonelliform brachiopods. However, due to small sample sizes, differing methodologies and comparisons involving brachiopods from different genera, it is difficult to fully quantify differences in growth between temperate and polar brachiopods. In order to achieve a direct growth comparison between a temperate brachiopod and an Antarctic brachiopod it is necessary to use similar methodologies in brachiopods that are closely related.

*Liothyrella neozelanica* is a temperate brachiopod that occurs in dense aggregations within Doubtful Sound, New Zealand (pers. obs.). Despite making up ecologically important habitat within New Zealand waters, the annual growth of *L. neozelanica* is largely undefined with one study describing adult specimens reaching a maximum length of 57 mm (Lee *et al.* 2001). By understanding the growth of *L. neozelanica*, insight into age, size, population structure and ecological importance can be gained. Furthermore, if similar methods are used to those described in Peck *et al.* (1997), a more robust comparison between temperate (*L. neozelanica*) and Antarctic brachiopods (*L. uva*) can be achieved using brachiopods from the same genus.

### 5.1.1 Chapter objective

1. The primary objective of this chapter is to determine the annual growth rate and population size structure of *Liothyrella neozelanica*
2. The secondary objective of this chapter is to compare the annual growth rates of *Liothyrella neozelanica* with *Liothyrella uva* to determine more robust conclusions about differences in growth and how these differences may be related to each brachiopod's specific surrounding environment.

## 5.2 Methods

### 5.2.1 Tagging

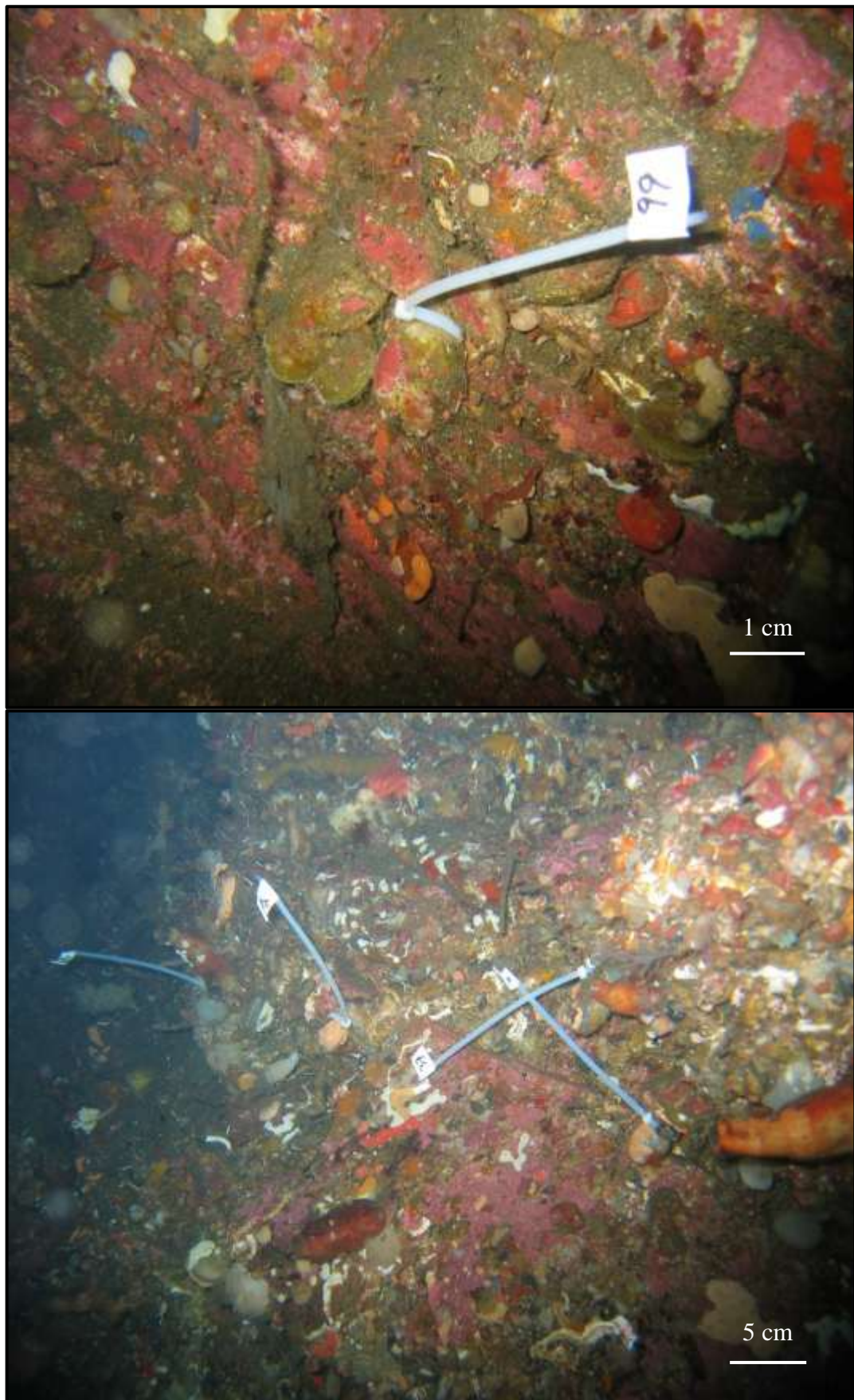
On the 25<sup>th</sup> January 2011, 101 *L. neozelanica* brachiopods were tagged *in situ* within Tricky Cove, Doubtful Sound using SCUBA. Cable ties marked with numbers were attached to the pedicles of *L. neozelanica* brachiopods at depths ranging between 15 to 23 meters (Fig. 5.1). The maximum width (Ref. Chap. 2, Sec. 2.2.1) of each tagged brachiopod was measured and recorded using vernier callipers to the nearest 0.5 mm. Previous growth studies involving brachiopods measured the length (mm) during tagging as opposed to measuring the width (Thayer 1977; Doherty 1979; Ostrow 2007). Due to *L. neozelanica* occurring in gregarious clumps within tight spaces, the true length of individuals was difficult to obtain and therefore the width was measured during this study. Once tagged and measured the brachiopods were left undisturbed for 12 months until the 7<sup>th</sup> December 2011, when the width of each tagged brachiopod was re-measured to the nearest 0.5 mm. A total of 60 individuals were re-identified with 41 individuals not recovered.

In order to achieve growth rate comparisons between studies, 581 individuals collected over the study period (December 2010 – December 2011) were measured to the nearest 0.01 mm using electronic vernier callipers (Ref. Chap. 2 Sec. 2.2.1) and the morphometric relationships between shell width (mm), length (mm) and height (mm) were established using the allometric regression equation  $y = ax^b$ . This equation was used to convert the initial and final width (mm) measurements of each tagged individual into length (mm).

### 5.2.2 Tetracycline tagging

In November 2010, *Liothyrella neozelanica* individuals were chemically tagged *in situ* within Tricky Cove at 18 meters depth with the fluorescent dye, tetracycline hydrochloride (TC) (657.5 mg / L). Plastic bags were attached to *L. neozelanica* individuals, injected with TC and left for 24 hours to ensure uptake. Furthermore, a sample *L. neozelanica* was collected after 24 hours and observed under UV (365 nm) light to confirm the uptake of TC (Appendix 3.1). Although the sampled *L. neozelanica* confirmed the uptake of TC (Appendix 3.1), individuals collected 13 months later in December 2011 showed no evidence of TC when observed under UV (365 nm) light. As a result, growth rates from TC tagging were unobtainable.





**Figure 5.1** Tagged and measured *Liothyrella neozelanica* brachiopods within Tricky Cove, Doubtful Sound.

### 5.2.3 Population structure

On the 23<sup>rd</sup> January 2012, the population size structure of *L. neozelanica* within Tricky Cove was determined by measuring the width (to the nearest 0.5 mm) of every *L. neozelanica* individual occurring within three square meters at 18 meters depth. A total of 293 individuals ranging from 0.5 mm to 45 mm were measured. A size-distribution plot was created and normal distributions were fitted using MIX 2.3 to determine different age cohorts of *L. neozelanica* within Tricky Cove.

### 5.2.4 Growth modelling

Two growth models were used within this study: 1) The Brody-Bertalanffy growth model; 2) An inverse logistic growth model.

#### *Brody-Bertalanffy growth model*

The Brody-Bertalanffy growth model is the most commonly used model in growth studies due to the ease at which it can be fitted to growth data from tagging experiments (Haddon *et al.* 2008). Furthermore, due to it being previously used in brachiopod studies (Peck *et al.* 1997; Ostrow 2007), it enables direct comparisons between brachiopod species.

The Brody-Bertalanffy (von Bertalanffy 1938; Brody 1945) growth model is defined as:

$$S_t = S_{\infty} (1 - be^{-Kt})$$

where  $S_t$  = size at time (mm),  $S_{\infty}$  = maximum size (mm),  $b$  = scaling parameter for size  $\neq 0$  at time 0,  $K$  = growth constant and  $t$  = time between initial and final measurements (years). A Walford regression plot ( $y = c + mx$ ) between initial and final shell length (mm) was used together with equations described in Lamare and Mladenov (2000) to determine the Brody-Bertalanffy parameters:

The maximum size ( $S_{\infty}$ ) was calculated as:

$$S_{\infty} = \frac{c}{(1 - m)}$$

The growth constant ( $K$ ) was calculated as:

$$K = \frac{-\ln(m)}{t}$$

The scaling parameter ( $b$ ) was calculated as:

$$b = 1 - \frac{S_0}{S_{\infty}}$$

where  $S_0$  = size at settlement. Chuang (1994) stated that *L. neozelanica* pre-settlement larvae ranged in length from 180 to 230 ( $\mu\text{m}$ ) and had a short planktonic stage before settling. Furthermore, Lee *et al.* (2010) stated that newly settled *Calloria inconspicua* and *Notosaria nigricans* brachiopods had a visible shell length of 250  $\mu\text{m}$ . From these findings the  $S_0$  of *L. neozelanica* was estimated in this study as 200  $\mu\text{m}$ .

#### *Inverse logistic regression growth model*

The inverse logistic model was used to avoid Brody-Bertalanffy model assumptions of rapid or slow growth within juveniles (Haddon *et al.* 2008). Furthermore, due to the Brody-Bertalanffy growth model being based on a linear relationship (Walford plot), the inverse logistic model is able to better fit data and provide a more realistic estimate of growth within *L. neozelanica* at different ages (Haddon *et al.* 2008). The inverse logistic growth model used within this study is defined as:

$$\Delta L = \frac{\max \Delta L}{1 + e^{\ln(19) \frac{(L_t - L_{50})}{(L_{95} - L_{50})}}}$$

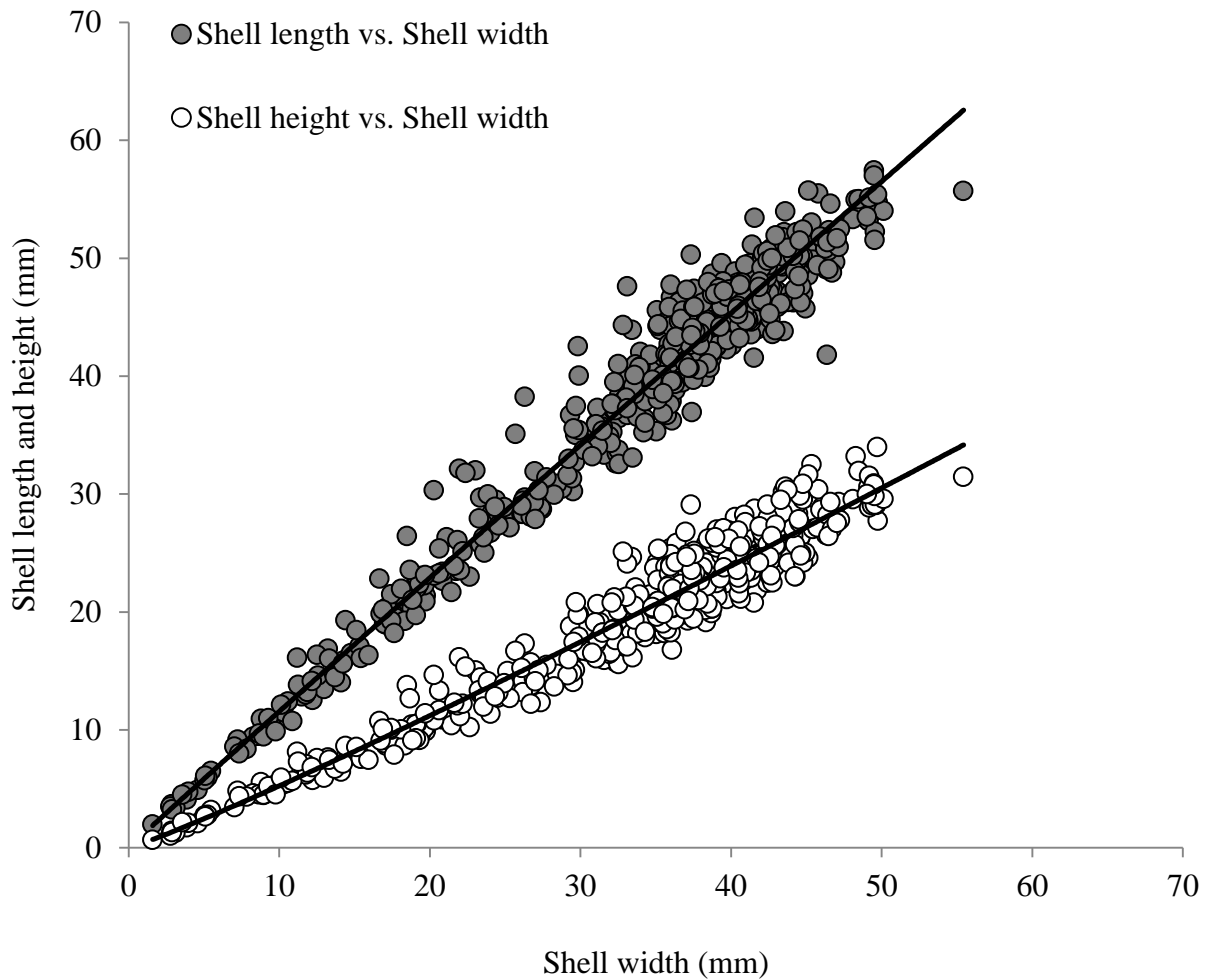
where  $\Delta L$  = change in length,  $\max \Delta L$  = maximum change in length,  $L_t$  = initial length,  $L_{50}$  = initial length when the midway point between  $\max \Delta L$  and lowest growth is reached and  $L_{95}$  = initial length when 95% of the difference between  $\max \Delta L$  and lowest growth is reached.

The parameters  $\max \Delta L$ ,  $L_{50}$  and  $L_{95}$  were estimated by fitting a best fit inverse logistic regression to a plot of growth increment (over 1 year) (mm) versus initial length (mm).

### 5.3 Results

#### 5.3.1 Morphometry of *Liothyrella neozelanica*

Morphometric relationships between shell length (mm), width (mm) and height (mm) of *Liothyrella neozelanica* are shown below (Fig. 5.2). Shell length was significantly ( $p < 0.0001$ ) related to shell width by the equation  $y = 1.2x^{0.99}$  ( $R^2 = 0.98$ ) (Fig. 5.2). Shell height was significantly ( $p < 0.0001$ ) related to shell width by the equation  $y = 0.42x^{1.09}$  ( $R^2 = 0.97$ ) (Fig. 5.2).



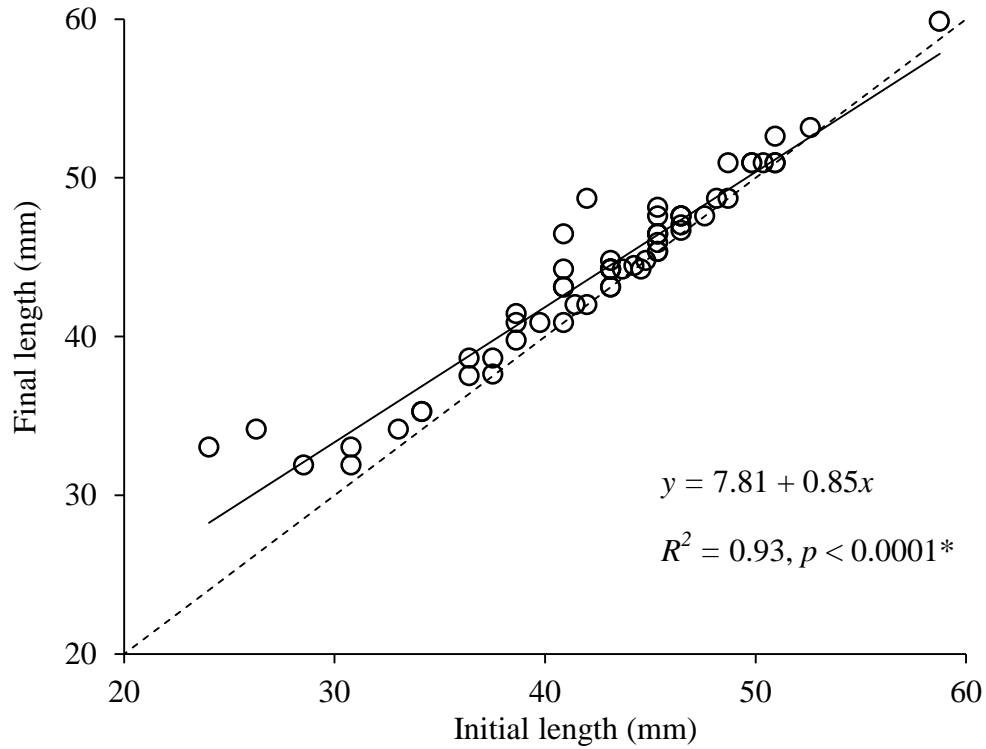
**Figure 5.2** Shell morphometry of *Liothyrella neozelanica*. Length (mm) vs. width (mm) is fitted with the allometric equation  $y = 1.2x^{0.99}$  ( $n = 581$ ). Height (mm) vs. width (mm) is fitted with the allometric equation  $y = 0.42x^{1.09}$  ( $n = 581$ ).

### 5.3.2 Annual growth of *Liothyrella neozelanica*

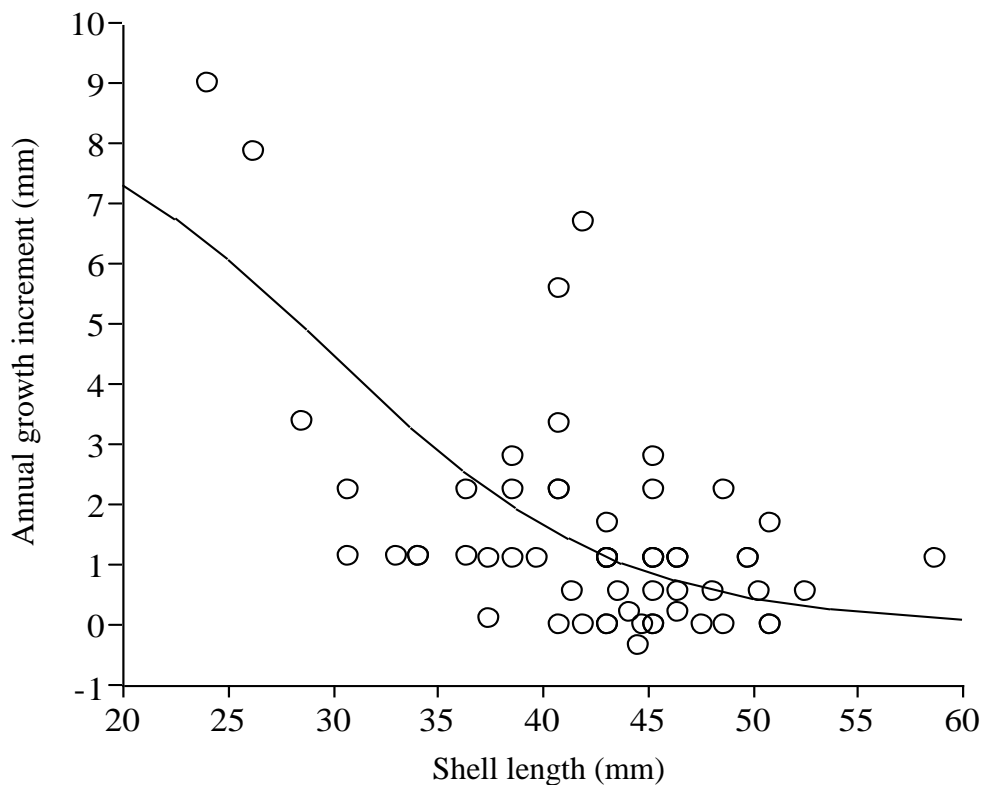
A Walford regression plot revealed a significant difference between the initial and final length measurements of tagged *Liothyrella neozelanica* ( $F = 754.6$ ;  $df = 1, 58$ ;  $p < 0.0001$ ) (Fig. 5.3 A). The fitted linear regression ( $y = 7.81 + 0.85x$ ) showed a distinctly different slope compared to that of a linear relationship representing no growth ( $y = x$ , slope = 1) (Fig. 5.3 A). The slopes of the fitted regression and the regression representing no growth intersected at 52 mm initial length (Fig 5.3 A). An inverse logistic regression fitted to a plot of annual growth vs. initial length revealed a best-fit when  $\max\Delta L$  was 9 mm,  $L_{50}$  was 30 mm and  $L_{95}$  was 50 mm (Fig. 5.3 B).

The growth constant (K) of *Liothyrella neozelanica* predicted by the Brody-Bertalanffy growth model was 0.16 with *L. neozelanica* reaching a maximum size ( $S_{\infty}$ ) of 52.5 mm in length at an age of 30 + years (Fig. 5.4 A). Maximum size at age 30 was predicted to be 52 mm length within the Brody-Bertalanffy growth model (SSE = 197,  $n = 60$ ) and 54 mm length in the inverse logistic growth model (SSE = 111,  $n = 60$ ) (Fig 5.4 A). The two models showed high initial growth rates before reaching an asymptote between 10 and 15 years of age (Fig. 5.4 A). The Brody-Bertalanffy growth model predicted relatively steady growth over the first 5 years of age, before declining between 5 and 10 years of age and plateauing at an age of 15 years (Fig 5.4 A). The inverse logistic model showed higher initial growth rates over the first 5 years of age compared to those rates predicted by the Brody-Bertalanffy model (Fig 5.4 A). Growth rates reached an asymptote between 7.5 and 10 years of age within the inverse logistic growth model (Fig. 5.4A).

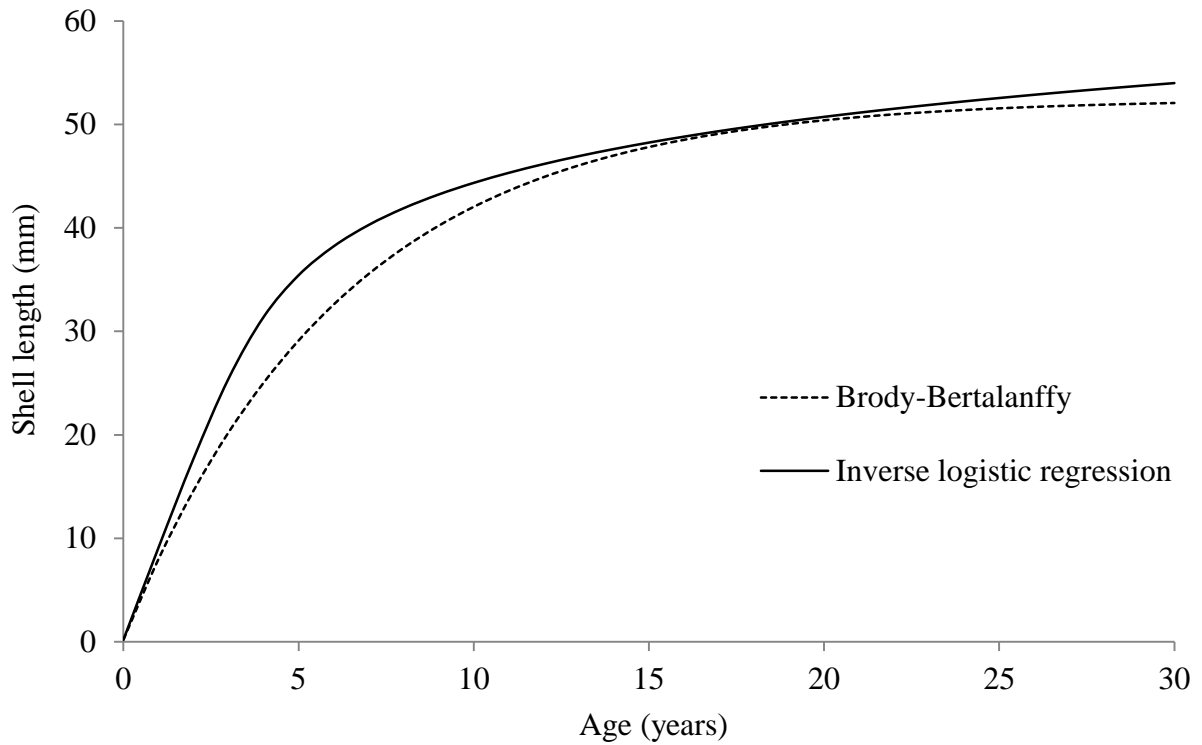
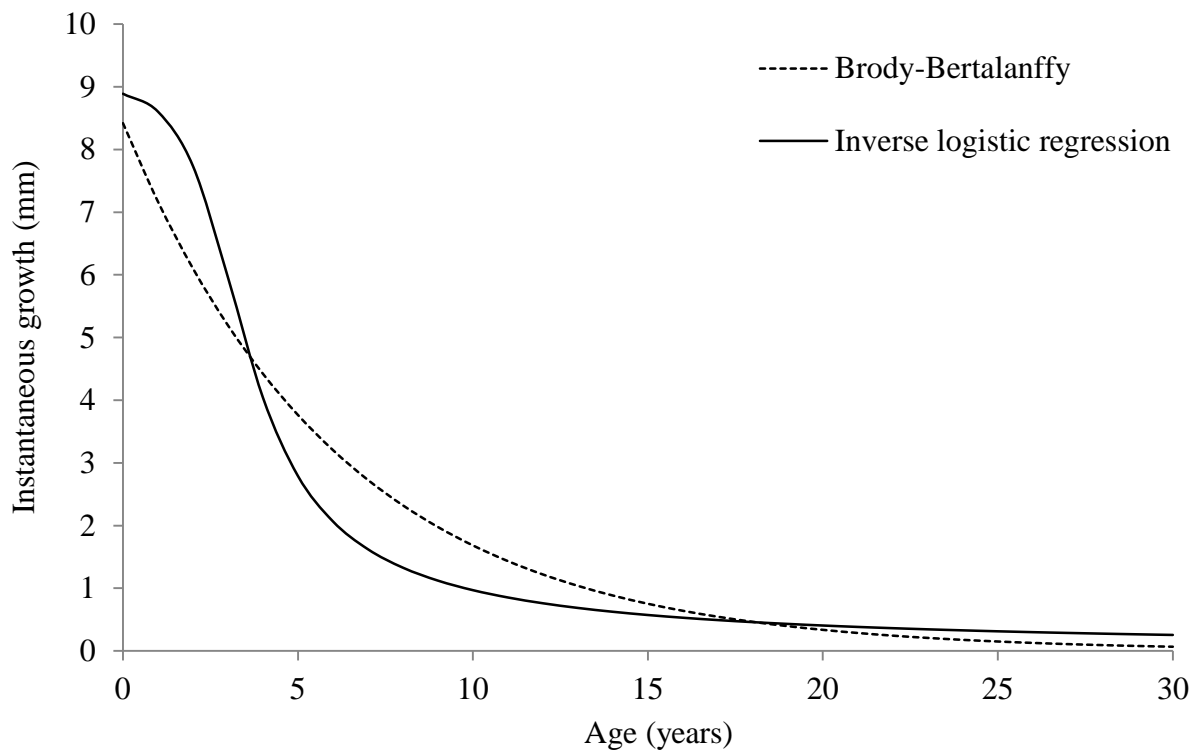
A.



B.



**Figure 5.3** (A) Walford plot of shell length (mm) for *L. neozelanica* over a 1 year period. Dotted 45° line represents zero growth. Solid line represents Brody-Bertalanffy regression line ( $n = 60$ ). (B) Shell length (mm) vs. growth increment (mm) fitted with an inverse logistic equation ( $\max \Delta L = 9$ ,  $L_{50} = 30$  and  $L_{95} = 50$ ) ( $n = 60$ ).

**A. Predicted growth****B. Instantaneous growth rate**

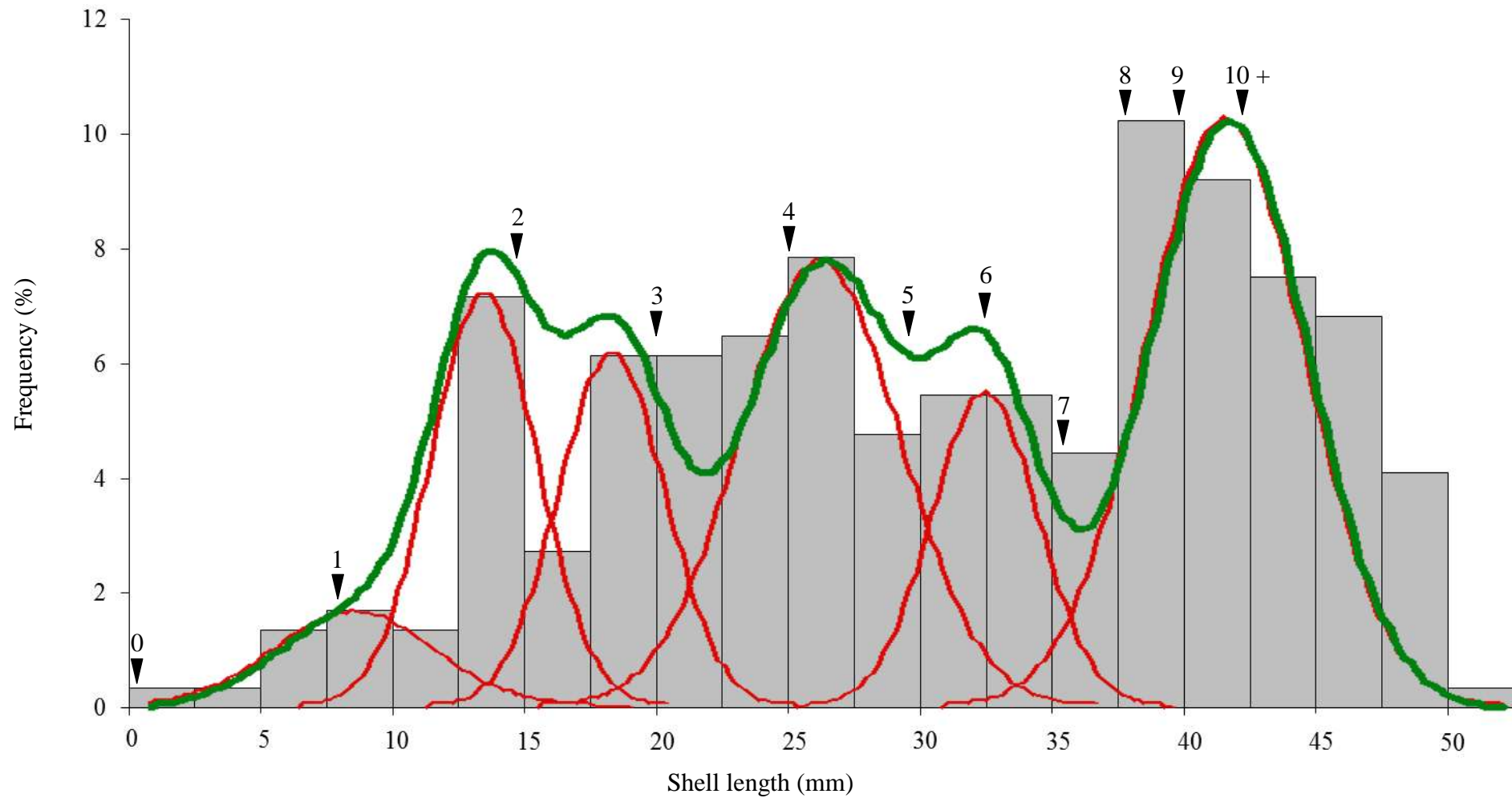
**Figure 5.4** (A) Predicted shell length (mm) at age (years) for *Liothyrella neozelanica* using Brody-Bertalanffy and inverse logistic growth models. (B) Predicted instantaneous growth of shell length (mm yr<sup>-1</sup>) at age (years) for *Liothyrella neozelanica* using Brody-Bertalanffy and inverse logistic growth models.

Predicted instantaneous growth rates were highest in individuals aged between 0 and 5 years in both models (Fig 5.4 B). Instantaneous growth upon settlement was  $8.9 \text{ mm yr}^{-1}$  within the inverse logistic growth model and  $8.4 \text{ mm yr}^{-1}$  within the Brody-Bertalanffy growth model (Fig. 5.4 B). Instantaneous growth within the Brody-Bertalanffy growth model steadily decreased as age increased (Fig. 5.4 B). Instantaneous growth predicted by the inverse logistic growth model remained relatively high ( $8.9 - 7.7 \text{ mm yr}^{-1}$ ) for the first two years of age, before decreasing to  $2.8 \text{ mm yr}^{-1}$  at 5 years of age (Fig. 5.4 B). Both models showed high instantaneous growth upon settlement and lower levels of instantaneous growth as age increased (Fig 5.4 B).

### 5.3.3 Population structure

The majority of the sampled population consisted of individuals greater than 20 mm in length (79%) with a recorded minimum of 1.2 mm in length and a maximum of 50.5 mm in length (Fig. 5.5). Six distinct cohorts were determined with fitted normal distributions peaking at 8 mm, 13 mm, 18 mm, 26 mm, 32 mm and 42 mm respectively (Fig 5.5). Size distribution appeared relatively constant at sizes above 12.5 mm. Size frequency appeared to be skewed to the left with the occurrence of higher numbers of larger individuals ( $> 25 \text{ mm}$  length) compared to smaller individuals ( $< 25 \text{ mm}$  length) (Fig. 5.5). Peaks in size distribution coincided relatively well with age estimations (years) predicted by the Brody-Bertalanffy growth model (Fig 5.5).





**Figure 5.5** Size-frequency distribution of *L. neozelanica* individuals sampled from Tricky Cove, Doubtful Sound ( $n = 293$ ). Red lines represent normally distributed cohorts. Green line represents the best fit of the normally distributed cohorts. Black arrows and corresponding numbers represent Brody-Bertalanffy age estimates (years) based on shell length (mm).

## 5.4 Discussion

### 5.4.1 Annual growth of *Liothyrella neozelanica*

Observed mortality between the 25<sup>th</sup> of January and the 7<sup>th</sup> of December was  $\approx 40\%$  which is substantially higher than the mortality observed in the only previous brachiopod tagging experiment involving the Antarctic *Liothyrella uva*. Peck *et al.* (1997) described a natural mortality rate of  $< 2\%$  per year for *L. uva*. The comparatively high rate observed for *L. neozelanica* suggests increased mortality which may have resulted from difficult *in situ* tagging conditions leading to damaged or irritated individuals. As a result, the methodology used may need to be refined and developed in future studies to reduce the observed mortality impact. However, the difference in mortality may result from *L. neozelanica* experiencing greater competition for space in its environment compared to *L. uva*. The environment inhabited by *L. neozelanica* is occupied by several species of competing Rhynchonelliform brachiopods including *Notosaria nigricans*, *Terebratella sanguinea*, *Calloria inconspicua* and *Neoemula vector* (Lee *et al.* 2010). As a result, competition for space is high and *L. neozelanica* is often colonised by competing species. This colonisation and competition for space may cause the comparatively high mortality observed for *L. neozelanica* compared to *L. uva*. Further research on brachiopod interactions and competition would provide insight into the observed differences in mortality.

The Brody-Bertalanffy and inverse logistic growth models both predicted high annual growth rates in juvenile *L. neozelanica* with declining growth rates as age increased. Maximum growth rate was exhibited on initial settlement (age 0) estimated at  $8.4 \text{ mm yr}^{-1}$  and  $8.9 \text{ mm yr}^{-1}$  by the Brody-Bertalanffy and inverse logistic growth models respectively. Faster growth rates in juvenile marine invertebrates compared to their adult counterparts is relatively common and has been noted in the temperate Rhynchonelliform brachiopods *Terebratalia transversa* (Thayer 1977), *Terebratulina retusa* (Collins 1991), *Calloria inconspicua* (Doherty 1979) and *Terebratella sanguinea* (Ostrow 2007). The fast growth of juveniles within these species is thought to occur in order to avoid mortality factors such as disturbance, predation and over-growth, by obtaining a size refuge at an early age (Thayer 1977; Doherty 1979; Collins 1991).

*Liothyrella neozelanica* larvae typically settle close to conspecific adults, often colonising the shells of already established individuals (Lee *et al.* 2010). Due to this, *L. neozelanica* often occur in gregarious clumps where growth can be distorted within individuals (pers. obs). Therefore, it is reasonable to suggest that rapid growth exhibited by juvenile *L. neozelanica* may be a direct response to over-growth by other brachiopods, where a size refuge is required to accommodate this. Furthermore, predation by amphipods and smothering by bryozoans and sponges has been noted within *L. neozelanica* populations, occasionally causing mortality (Lee 2008; Lee *et al.* 2010; pers. obs.). Rapid growth in juveniles may enable the pedicle to develop quickly, thereby lifting the brachiopod away from the substrate to minimise contact with predators and encrusting organisms and to enable access to the water column for feeding.

In addition, the rapid growth in juvenile *L. neozelanica* and relatively slow growth in their adult counterparts may be a direct consequence of individuals reaching reproductive maturity, whereby energy is re-allocated from somatic growth to gonadal development. Chuang (1994) observed mature gonads within a female *L. neozelanica* measuring 22 mm in length and immature gonads with indeterminate sex in an individual 20 mm in length. These findings suggest that the development of gonads within *L. neozelanica* begins when individuals reach lengths of approximately 20 mm, reaching maturity at a length of > 22 mm. These *L. neozelanica* lengths (20 mm and 22 mm) correspond to an age of 2 to 3 years based on the Brody-Bertalanffy and inverse logistic growth models. Interestingly, growth rates at this age are still estimated to be 7 to 5 mm yr<sup>-1</sup>, suggesting that growth still occurs at relatively high rates irrespective of gonadal development at this time. This may be a reflection of the energy requirements for gonad development whereby smaller gonads within smaller individuals provide less of a drain on energy normally utilised for growth. These findings further support the theory of a size-refuge playing an integral role in the early life history of *L. neozelanica*, although it is likely that reproductive development plays a key role in slowing somatic growth in adult individuals. Further research is needed to investigate this.

On comparison with previous studies involving temperate Rhynchonelliform brachiopods, *L. neozelanica* has a comparable growth constant ( $K = 0.16$ ) with the lower growth constant described for *Terebratella sanguinea* ( $K = 0.13$ ) (Ostrow 2007) (Table 5.1). Furthermore, the maximum growth rate is similar to that described for *Terebratella sanguinea* (Ostrow 2007), and *Terebratalia transversa* (Thayer 1977) at around 8 mm yr<sup>-1</sup>, possibly signifying a

common maximum growth rate for temperate brachiopods belonging to the family Terebratulidae (Table 5.1). The maximum growth reported for *Calloria inconspicua* was 5 mm yr<sup>-1</sup> (Doherty 1979), suggesting that maximum growth rates are species-specific and most likely reflect the environmental conditions present within each respective habitat (Table 5.1).

Comparisons of the maximum sizes ( $S_{\infty}$ ) of Rhynchonelliform brachiopods, show *L. neozelanica* is able to achieve a maximum size (52.5 mm) that is almost double that achieved by *Terebratella sanguinea* and 2.4 times greater than that achieved by *Calloria inconspicua* (Table 5.1). This difference in maximum size is also reflected in maximum age as *L. neozelanica* is able to reach a maximum age (30+ years) that is 10 to 16 years older than those described for *Terebratella sanguinea* and *Calloria inconspicua* respectively (Table 5.1). These findings suggest that *L. neozelanica* is relatively long-lived compared to other temperate brachiopods. However, there is limited information on the effects of environmental conditions and habitat stability on the growth of Rhynchonelliform brachiopods making it difficult to understand the observed differences in growth. Further research into the effects of ambient factors (water temperature, site productivity) on growth is needed, preferably using similar methodologies to previous studies, in order to clarify these issues.

**Table 5.1** Growth comparisons between *Liothyrella neozelanica* ( $n = 60$ ), *Terebratella sanguinea* ( $n = 24$ ), *Calloria inconspicua* ( $n = 827$ ) and *Terebratalia transversa* ( $n = 226$ ) (Growth parameters estimated from von-Bertalanffy (Ostrow 2007) and Brody-Bertalanffy (present study) growth models:  $K$  = growth constant,  $S_{\infty}$  = maximum size) (*Terebratalia transversa* and *Calloria inconspicua* growth parameters determined observationally).

Species	$K$	Max. growth rate (mm yr <sup>-1</sup> )	$S_{\infty}$ (mm)	Max. age (years)	Study
<i>Liothyrella neozelanica</i>	0.16	8.42	52.5	30 +	Present
<i>Terebratella sanguinea</i>	0.13 – 0.46	4 - 12.8	28.15 – 30.88	20 +	Ostrow (2007)
<i>Calloria inconspicua</i>	-	5	22	14 +	Doherty (1979)
<i>Terebratalia transversa</i>	-	8	-	-	Thayer (1977)

### 5.4.2 Population structure

The sampled *L. neozelanica* population within this study was made up primarily of large individuals (> 25 mm length) with few juveniles (< 10 mm) being observed. As a consequence the size-frequency distribution of *L. neozelanica* was skewed to the left. This is in contrast to the majority of brachiopod studies which show either right-skewed size-frequency distributions with high levels of juveniles or bi-modal size-frequency distributions containing high numbers of both juvenile and adult individuals (Thayer 1977; Doherty 1979; Collins 1991; Brey *et al.* 1995). A possible explanation for this trend may be that juveniles were under-represented due to difficulties in measuring *in situ* at 18 meters depth using SCUBA.

However, left-skewed size frequency distributions have been observed for other marine invertebrate species including the mollusc *Halliotis iris* (Sainsbury 1982) and also in the Antarctic brachiopod *Magellania fragilis* (Brey *et al.* 1995). The left-skewed size-frequency distribution is thought to occur in marine invertebrates as a result of either: 1) episodic recruitment events; 2) low mortality rates compared to growth rates, causing high numbers of larger, older individuals; 3) or mortality being size-dependent with increased size creating a refuge from major mortality factors (Sainsbury 1982; Brey *et al.* 1995). From the high growth rate estimates predicted by the Brody-Bertalanffy and inverse logistic growth models for juvenile *L. neozelanica* (Refer Sec. 5.4.1), it may be possible that a size-refuge exists for *L. neozelanica* individuals. Furthermore, Lee *et al.* (2010) observed a 96% mortality rate for juvenile *L. neozelanica* over the first 32 months of settlement despite high numbers of new recruits, again suggesting a size-refuge is required by juveniles. Finally Peck (1993) found that predation on adult brachiopods is likely to be low due to the internal tissues being low in density and containing high levels of inorganic material. This finding may mean that *L. neozelanica* have low mortality rates once a size refuge is achieved, enabling high numbers of large, old individuals within a population. These three findings provide evidence to support the left-skewed size-distribution of the sampled *L. neozelanica* population observed within this study.

There were six main cohorts shown in the size-frequency distribution from a sampled population of 293 *L. neozelanica* individuals in this study. The peaks of these cohorts lined up relatively well with consecutive age (years) estimations derived from the Brody-Bertalanffy growth model suggesting a single annual recruitment. Lee *et al.* (2010) found evidence of multiple recruitment events of *L. neozelanica* taking place in February-March and in spring over a 21 month period, due to the presence of juveniles ( $\leq 2.5$  mm length) on settling plates. Due to the modes in the size-frequency distribution of this study not being readily distinguishable, it is unclear whether single or multiple recruitment episodes may be taking place.

Multiple recruitment episodes in temperate Rhynchonelliform brachiopod populations are not uncommon with Thayer (1977) finding evidence of three recruitment episodes occurring over an annual period for *Terebratalia transversa* and Doherty (1979) finding evidence of two recruitment episodes taking place over an annual period for *Calloria inconspicua*. However, it must be noted that brachiopods of the same age can exhibit large variation in growth rates; obtaining different sizes at different ages (Thayer 1977). Therefore, although there is evidence of multiple recruitment throughout an annual period for *L. neozelanica* (Lee *et al.* 2010), it is possible that *L. neozelanica* is recruiting once annually and variation in growth rates amongst individuals is creating the observed difficulty in readily distinguishing modes within the size-frequency distribution.

Furthermore, evidence of only one annual *L. neozelanica* spawning event occurring between July and September 2011 was presented in chapter 4, which appears to be represented by the presence of newly settled juveniles ( $< 5$  mm length) within the size-frequency distribution. If spawning between July and September is correct and the Brody-Bertalanffy growth estimates are correct (Age 0 = 0.2 mm length, instantaneous growth =  $8.4 \text{ mm yr}^{-1}$ ; Age 1 = 7.8 mm length, instantaneous growth =  $7.2 \text{ mm yr}^{-1}$ ) then individuals spawned in August 2011 (Age = 5 months) would be on average 3.4 mm in length and individuals spawned in August 2010 (Age = 17 months) would achieve an average size of 10.3 mm in length. These two size classes are both represented within the size-frequency distribution again suggesting one recruitment episode throughout an annual period. However it must be noted that these findings are based upon one size-frequency survey and therefore further research, including

multiple size-frequency surveys and specific growth rates for juvenile *L. neozelanica* (0.2 - 25 mm length) are needed in order to clarify this issue.

### 5.4.3 *Liothyrella* comparisons

Growth comparisons between *Liothyrella neozelanica* and *Liothyrella uva* are shown in Table 5.2. The maximum growth rate of a 5 mm long *L. uva* individual is 3.3 to 4.7 times slower than that of a 5 mm long *L. neozelanica* individual and growth within a 20 mm long *L. uva* individual is 3.7 to 5.4 times slower than the rate exhibited by a 20 mm long *L. neozelanica* individual (Table 5.2). Peck *et al.* (1997) described *L. uva* as having growth rates that ranged from 2 to 6 times slower than those observed in the temperate brachiopods *Terebratalia retusa* (Collins 1991) and *Calloria inconspicua* (Doherty 1979). These findings support the conclusion that *L. uva* has comparatively slow growth rates compared to temperate brachiopods, with *L. neozelanica* having growth rates 3.3 to 5.4 times higher than those described for *L. uva*.

**Table 5.2** Growth comparisons between the temperate brachiopod *Liothyrella neozelanica* ( $n = 60$ ) and the Antarctic brachiopod *Liothyrella uva* ( $n = 324$ ) (Parameters estimated from a von-Bertalanffy growth model (Peck *et al.* 1997) and a Brody-Bertalanffy growth model (present study): K = growth constant,  $S_{\infty}$  = maximum size).

Species	K	Max. growth (5 mm) (mm yr <sup>-1</sup> )	Max. growth (20 mm) (mm yr <sup>-1</sup> )	( $S_{\infty}$ ) (mm)	Max. age (years)
<i>Liothyrella neozelanica</i>	0.16	7.5	5.19	52.5	30+
<i>Liothyrella uva</i>	0.03 – 0.05	1.6 – 2.3	0.96 – 1.4	47.2 – 54.1	46 - 71

Traditionally cold temperatures were considered the major factor influencing slow growth in Antarctic marine invertebrates; however, new evidence suggests that seasonal resource limitation is the major cause (Clarke 1991; Peck *et al.* 2002). Barnes (1995) and Peck *et al.* (2000) found consistent seasonal growth in Antarctic bryozoans and in the Antarctic bivalve *Yoldia eightsi* respectively, suggesting that growth in Antarctic marine invertebrates may be independent of seasonal factors. There is still much debate over what factors cause the slow growth of marine invertebrates in the Antarctic environment and therefore it is unclear what factors may be causing the observed difference in growth between *L. neozelanica* and *L. uva*. It is likely that growth in both *L. neozelanica* and *L. uva* is governed by a combination of abiotic and biotic factors within each respective environment. Further research is needed to clarify this issue.

Interestingly, despite the maximum growth rates of *L. uva* being lower than those described for *L. neozelanica*, the maximum size of *L. uva* is similar to that described for *L. neozelanica* (Table 5.2). As a result, the maximum age of *L. uva* ranges from 46 to 71 years old whereas the maximum age described for *L. neozelanica* is around 30 years (Table 5.2). Slow growth rates, coupled with relative longevity may make *L. uva* more susceptible to climate change compared to temperate counterparts, as population turnover rates may not be fast enough to enable sufficient adaptation to relatively rapidly changing environmental conditions.

#### 5.4.4 Conclusion

Initial growth rates of *Liothyrella neozelanica* juveniles are relatively high from initial settlement ( $8 \text{ mm yr}^{-1}$ ) to lengths of 20 mm ( $5 \text{ mm yr}^{-1}$ ), suggesting juveniles may need a size refuge in order to avoid mortality factors. Slowing growth rates as individuals reach shell lengths greater than 25 mm may suggest re-allocation of energy from somatic growth to gonadal development as individuals become sexually mature. The size frequency distribution of a sampled population of *L. neozelanica* produced a left-skewed size frequency indicating the absence of high numbers of juveniles. This finding may reflect low predation rates in adult individuals and the presence of a size refuge, whereby juvenile *L. neozelanica* undergo high levels of mortality until reaching this refuge. Evidence of annual recruitment is shown within the size frequency distribution, with observed cohort distributions coinciding with age estimates predicted by a Brody-Bertalanffy growth model. However, it is still unclear whether



*L. neozelanica* undergoes a single recruitment event or multiple recruitment events throughout an annual period. Comparisons of *L. neozelanica* to the Antarctic *Liothyrella uva* show that *L. neozelanica* grows between 3 to 5 times faster than *L. uva*. It is unclear what factors may be causing these observed differences in growth between the two species. Although slow-growing, *L. uva* reaches a similar maximum size to that described for *L. neozelanica* and as a consequence attains ages well in excess of the maximum age described for *L. neozelanica*. Slow growth, coupled with a relatively long life-span may cause implications for *L. uva* in the future as climate change progresses.

# CHAPTER 6

## GENERAL DISCUSSION



*Liothyrella neozelanica* in Tricky Cove, Doubtful Sound, New Zealand (Photograph courtesy of Mike Barker).

**“It is good to have an end to journey toward;  
but it is the journey that matters, in the end”  
(Ursula K. LeGuin)**

## 6.1 General biology of *Liiothyrella neozelanica*

### *Seasonal metabolism*

The ambient respiration rate of *L. neozelanica* was investigated over an annual period to understand how it metabolically adjusts to changing environmental conditions. Ambient respiration rates ranged between 243.5 and 560  $\mu\text{g}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$  throughout the sampling period, suggesting *L. neozelanica* actively adjusts its metabolic rate to changing surrounding environmental conditions. It was determined that ambient sea temperature caused some of the observed variation (32%) in the ambient respiration rate of *L. neozelanica*, whereas ambient chlorophyll concentration caused the majority of the observed variation (78%). Respiration rates remained relatively steady throughout winter despite decreasing ambient sea temperatures, suggesting *L. neozelanica* thermally compensates to changes in temperature. It was determined that *L. neozelanica* has a broad thermal tolerance, exhibiting aerobic respiration at temperatures ranging from 8°C to 23°C. These findings may suggest *L. neozelanica* is well adapted to cope with a broad temperature range (8°C - 18°C) exhibited in its ambient environment (Lee 1991; Witman and Grange 1998). The main conclusions in regards to *L. neozelanica* respiration are:

- *L. neozelanica* metabolism is positively influenced by ambient sea temperature and negatively influenced by ambient chlorophyll concentration
- *L. neozelanica* shows evidence of metabolic thermal compensation to seasonal changes in ambient sea temperature
- *L. neozelanica* exhibits evidence of a broad metabolic thermal tolerance range

*Seasonal biochemistry*

Soluble protein, lipid and carbohydrate levels were determined within the internal tissues of *L. neozelanica* over an annual period to understand the seasonal energy requirements of *L. neozelanica*. Soluble protein levels occurred in the greatest amounts and showed the greatest seasonal variation in the internal tissues of *L. neozelanica* throughout the sampled months. In contrast carbohydrate levels were minimal suggesting soluble protein is the major energy substrate utilised by *L. neozelanica*. Furthermore, lipid was found to be the major biochemical found within the gonad of *L. neozelanica*, steadily increasing across the sampling period, peaking in September 2011 and declining in the months that followed. These findings suggest that *L. neozelanica* may rely on protein as the major energy source for processes such as metabolism, growth and reproductive development whereas lipid is likely the major energy store within the gametes of *L. neozelanica*, utilised by the lecithotrophic larvae during development. Furthermore, the September peak in gonad lipid levels, followed by a significant reduction in October 2011 suggests that a spawning event may have occurred throughout this time. The main conclusions of *L. neozelanica* seasonal biochemistry are:

- Protein appears to be utilised as the major energy substrate within the internal tissues of *L. neozelanica*
- Lipid appears to be the major energy store within the gonads of *L. neozelanica* during reproductive development
- Significant reduction in gonadal lipid between September 2011 and October 2011, may suggest a spawning event

*Annual reproduction*

The reproductive cycle of *L. neozelanica* was investigated over an annual period to determine the spawning cycle, as previous findings are inconclusive with spawning being observed in March / April (Tortell 1981; Chuang 1994) and spring (Lee *et al.* 2010). Gametogenic evidence found throughout the sampled months suggests *L. neozelanica* spawned between July 2011 and September 2011 (spring). High numbers of mature oocytes in July followed by high numbers of immature oocytes in September, together with the presence of necrotic tissue and degrading lamella in October 2011 support this finding. A spawning episode agrees with previous observations of larval recruitment by Lee *et al.* (2010), but disagrees with spawning observations described by Tortell (1981) and Chuang (1994) who observed spawning of individuals in the laboratory between February and March. The presence of mature oocytes throughout the sampled months, may suggest that *L. neozelanica* spawns multiple times throughout an annual period which may explain the variation in spawning observations (Tortell 1981; Chuang 1994). Negative correlations between ambient photoperiod, ambient chlorophyll concentration and oocyte size suggests spawning in *L. neozelanica* may be queued to these environmental parameters whereby increasing photoperiod and chlorophyll concentrations in spring trigger spawning. The main conclusions of *L. neozelanica* reproduction are:

- Spawning suggested to occur between the end of winter (July) and the start of spring (September)
- Evidence of mature oocytes throughout an annual period suggests *L. neozelanica* may undergo multiple spawning events throughout an annual period
- *L. neozelanica* spawning likely cued to ambient photoperiod and ambient chlorophyll concentration

### Growth

Annual growth was investigated for *L. neozelanica* over an annual period to gain a greater understanding of the population dynamics, age and life-history of *L. neozelanica*. The maximum growth rate of *L. neozelanica* was estimated at 8.4 mm yr<sup>-1</sup> to 8.9 mm yr<sup>-1</sup> by the Brody-Bertalanffy and inverse logistic growth models respectively. Growth rates were highest in juvenile *L. neozelanica*, slowing as individuals matured. The comparatively rapid growth in juveniles compared to adults may represent the need for a size-refuge by *L. neozelanica* individuals whereby predation, over-growth and restricted access to the water column are overcome by juveniles exhibiting rapid growth. The presence of a size-refuge for *L. neozelanica* was further supported by the lack of juveniles within a population size-frequency distribution which was made up primarily of individuals greater than 25 mm in length. Age estimations based on the Brody-Bertalanffy growth model lined up well with cohorts identified within the population size-frequency distribution, suggesting annual recruitment. However, sampling within the size-frequency distribution was coarse and therefore it is difficult to determine whether single or multiple recruitment episodes are occurring throughout an annual period. Further research is required to solve this issue. The main conclusions for *L. neozelanica* growth are:

- Evidence suggests juvenile *L. neozelanica* may require a size-refuge to avoid mortality
- Populations of *L. neozelanica* appear to be made up primarily of adult individuals (> 25 mm)
- Evidence suggests annual recruitment of *L. neozelanica*

## 6.2 *Liothyrella* comparisons

Comparisons between the temperate Rhynchonelliform brachiopod *Liothyrella neozelanica* and the Antarctic Rhynchonelliform brachiopod *Liothyrella uva* are shown in Table 6.1. The summer and winter metabolic rates of *L. neozelanica* are 1.9 times and 2.9 times higher than those rates exhibited by *L. uva* under simulated summer and winter conditions after a Q<sub>10</sub> correction is applied (Table 6.1). This finding suggests *L. uva* may have cold-adapted its metabolism in response to the cold temperatures and extreme seasonality in productivity, within the Southern Ocean, which agrees with previous findings by Peck *et al.* (1986) and Peck (1996).

Biochemical comparisons reveal that both *L. neozelanica* and *L. uva* rely on protein as a major energy substrate with carbohydrate occurring in relatively minor quantities within internal tissues (Table 6.1). Furthermore, both species utilise lipid as a major energy source during reproductive development (Table 6.1). The similar use of biochemicals may represent a historical link between the two species. Interestingly, the quantities of each biochemical were notably greater within the internal tissues of *L. uva* compared to *L. neozelanica*, suggesting *L. uva* may store higher quantities of each biochemical as reserves to be used in over-wintering the relatively unproductive winters in the Antarctic environment.

Both *L. neozelanica* and *L. uva* were found to spawn in the Austral spring. Although it is unknown what environmental cues may trigger reproductive development and spawning in *L. uva*, it is likely that spawning of this species coincides with increased food availability. In this study photoperiod and ambient chlorophyll concentration were found to be significantly correlated to the reproductive development and spawning in *L. neozelanica*. Due to both species spawning in spring, it may be possible that photoperiod and chlorophyll concentration may also be responsible for reproductive development and spawning within *L. uva*.

**Table 6.1** Metabolic, biochemical, reproductive and growth comparisons between the temperate Rhynchonelliform brachiopod *Liothyrella neozelanica* and the Antarctic Rhynchonelliform brachiopod *Liothyrella uva*.

Research	Parameter	<i>Liothyrella neozelanica</i>	<i>Liothyrella uva</i>	<i>Liothyrella uva</i> study
Metabolism	Summer oxygen consumption ( $\mu\text{g}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$ )	89	46	Peck <i>et al.</i> 1986
	Winter oxygen consumption ( $\mu\text{g}(\text{O}_2) \text{ g}^{-1}(\text{AFDM}) \text{ hr}^{-1}$ )	109.5	38	
Biochemistry	Major energy substrate	Protein	Protein	Peck <i>et al.</i> 1987
	Minor energy substrate	Carbohydrate	Carbohydrate	
	Energy substrate used in reproductive development	Lipid	Lipid	
Reproduction	Spawning period	July - September	October – November	Meidlinger <i>et al.</i> 1998
Growth	Growth constant	0.16	0.03 – 0.05	Peck <i>et al.</i> 1997
	Max. growth (5 mm individual) (mm yr <sup>-1</sup> )	7.5	1.6 – 2.3	
	Max. age (years)	30 +	46 – 71	
	Max. size (length mm)	52.5	47.2 – 54.1	



Growth results reveal *L. neozelanica* to grow 3 to 5 times faster compared to *L. uva* individuals of similar size, despite both species reaching a similar maximum size (Table 6.1). It is unclear what environmental factors may be affecting the observed differences in growth between the two species, however it is likely that the extreme seasonality in primary productivity within the Southern Ocean restricts the amount of energy that *L. uva* can invest in growth throughout an annual period. Due to *L. uva* reaching a similar size to *L. neozelanica* despite slower growth rates, *L. uva* attains a greater maximum age compared to *L. neozelanica* (Table 6.1). The slow growth of *L. uva*, coupled with a greater longevity may cause greater implications for *L. uva* compared to *L. neozelanica* in the future as climate change progresses.

### 6.3 Climate change implications

Climate change in the next century is predicted to warm the oceans on average by 2°C (IPCC 2007). The findings in this study suggest that *L. neozelanica* adult individuals will be largely unaffected by a 2°C change in ambient sea temperature. Adult *L. neozelanica* adults exhibited a broad thermal tolerance (eurythermic) and were able to aerobically respire at 23°C; 5°C above the ambient temperature range (8 - 18°C) experienced within Doubtful Sound. It was found that reproductive development within *L. neozelanica* is likely cued to ambient photoperiod and ambient chlorophyll concentration as opposed to ambient sea temperature. It seems unlikely that photoperiod will be affected as a result of a 2°C increase in sea temperature, however primary productivity may be affected if an increase in temperature causes increased stratification of the water column, thereby reducing the supply of nutrients to the euphotic zone (Harley *et al.* 2006). Due to the majority of marine invertebrates cueing reproduction to photoperiod (Lawrence and Soame 2004), it seems reasonable to suggest that the reproductive cycle of *L. neozelanica* will be largely unaffected by a direct increase in sea temperature, however decreased primary productivity may occur and further research is required to understand the reproductive effects this may incur. Furthermore, information regarding the effects of raised sea temperature on the development and mortality of *L. neozelanica* larvae is undefined and further research is needed to clarify the implications associated with this.

Evidence suggests that juvenile *L. neozelanica* require a size-refuge in order to avoid mortality and therefore exhibit higher growth rates compared to their adult counterparts. A

2°C increase in ambient sea temperature may affect growth rates of juvenile *L. neozelanica* as more energy is used to fuel higher metabolic rates. As a result, growth rates in juvenile *L. neozelanica* may be reduced, which may lead to increased mortality rates as individuals take longer to reach a size-refuge. Again further research is needed to clarify juvenile *L. neozelanica* growth rates to reveal the effects increased sea temperatures may have on this parameter.

Results from this study suggest that a 2°C increase in ambient sea temperature may have some effects on *L. neozelanica*, but it is likely that the Antarctic *L. uva* will experience greater effects. Slow metabolic rates (stenothermic) coupled with a narrow thermal tolerance may mean that *L. uva* is more vulnerable to a 2°C change in sea temperature within the Antarctic environment. Furthermore, *L. uva*'s comparatively slow growth rate and greater longevity compared to *L. neozelanica* may mean that *L. uva* will be more susceptible to increased sea temperatures as population turn-over rates may not be fast enough to allow sufficient adaption to relatively rapidly changing environmental conditions. The main conclusions for the effect of climate change on *Liothyrella* are:

- *L. neozelanica* will be able to metabolically tolerate a 2°C increase in ambient sea temperature over the next century
- *L. neozelanica* reproduction will be relatively unaffected by a 2°C increase in ambient sea temperature.
- The growth rates of juvenile *L. neozelanica* may be reduced, which in turn may lead to increased mortality rates as it takes longer to reach a size-refuge
- Evidence suggests the Antarctic *L. uva* may be more susceptible to climate change in the form of raised sea temperature, compared to its temperate counterpart *L. neozelanica*

#### 6.4 Suggestions for further research

The research presented has provided a starting block from which further research into the life-history of *L. neozelanica*, comparisons between temperate and polar brachiopods, and insight into climate change implications can be built. In order to fully quantify how *L. neozelanica* interacts with its environment and how it may be affected in the future towards increased sea temperatures, research on the larvae of *L. neozelanica* needs to be undertaken; specifically in regards to the effect of temperature on larval metabolism, growth rates and mortality rates. By understanding these parameters, further knowledge of the population dynamics and the general biology of *L. neozelanica* will be achieved, providing insight into how raised sea temperatures resulting from climate change may affect *L. neozelanica* and brachiopods in general over the next century.

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# Appendix 1

(Seasonal metabolism of *Liothyrella neozelanica*)

## Appendix 1.1

Before each collection within Tricky Cove (Ref. Chap. 2, Sec. 2.2.1), three closed tanks (60 cm x 37 cm x 26 cm) holding sea water from Otago Harbour were set up within a controlled temperate (CT) room at PML. Each tank was supplied with an aerator and fitted with a lid to avoid increased water salinities via evaporation. The first tank acted as a holding tank, housing the experimental brachiopods, and the second tank acted as a water reservoir for the first. Every second day, 90 to 95% of the water from the first tank was siphoned out and replaced with water from the second tank in order to minimise stress through waste build up. The third tank held 1µm filtered water which was used in the respiration experiments.

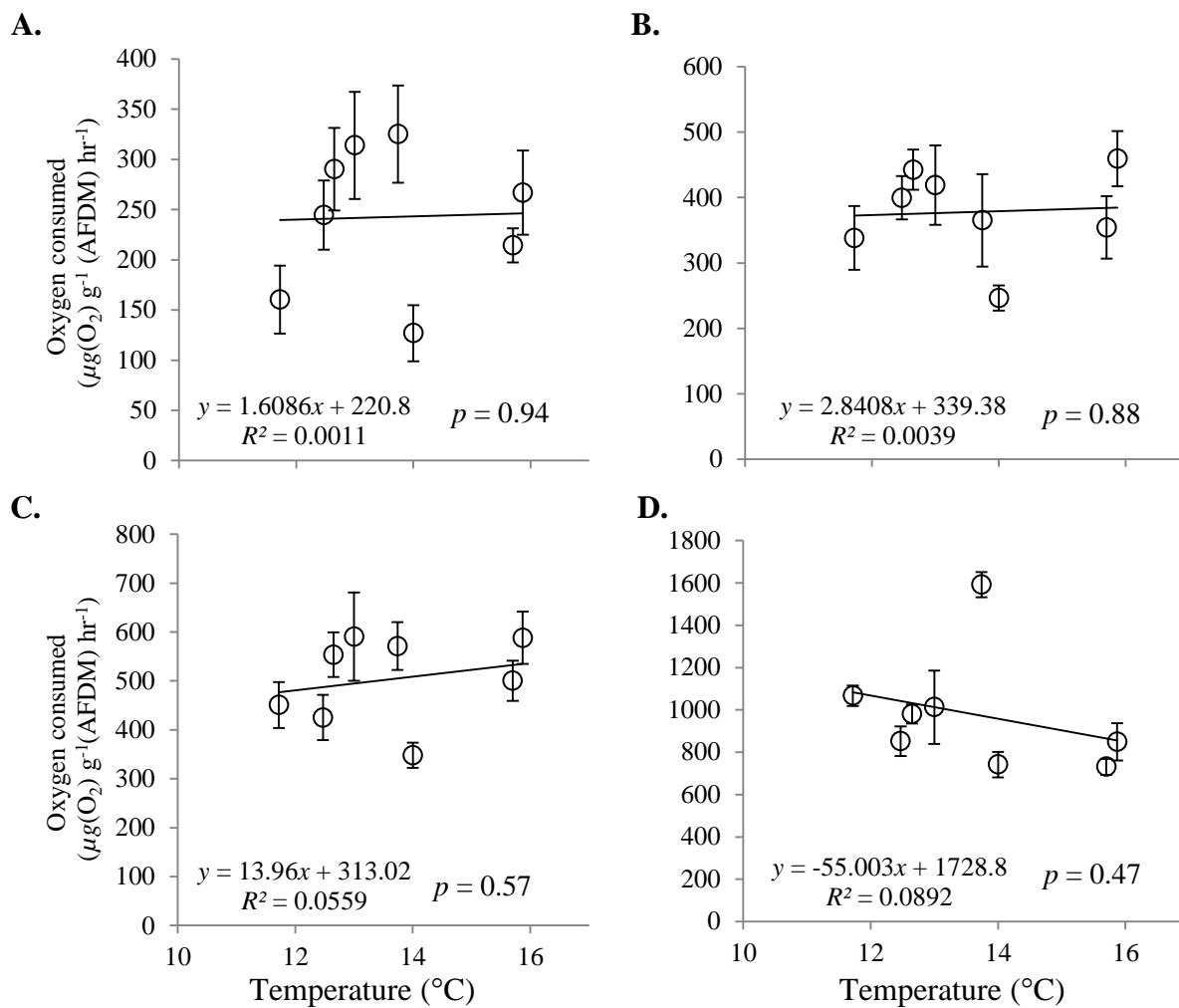
## Appendix 1.2

The temperature of the CT room was pre-set before each collection to an estimate of what the Tricky Cove ambient water temperature at 18 meters depth would be. This was done to minimise stress on the brachiopods during their introduction to the holding tank by reducing the difference in water temperatures found between Tricky Cove and the Otago Harbour supplied PML tanks. Temperature estimates differed by a maximum of 2°C to actual values recorded in Tricky Cove throughout the experimental period. Once the 18 meter water temperature within Tricky Cove was determined from a CTD profile, the tank temperature was adjusted accordingly to the nearest degree. Water temperatures within the CT room fluctuated within 0.4°C of the set temperature throughout the experimental period.

## Appendix 1.3

After 48 hours of recovery, the respiration rates at ambient temperature were determined. The brachiopods were then acclimated in descending order to the next coldest temperature for a minimum of 48 hours before being tested. To acclimatise brachiopods up to 18 and 23°C from 8°C, the temperature was adjusted by 5°C systematically every 48 hours until the experimental temperature was reached.

## Appendix 1.4



**Figure Appendix 1.4** The effect of ambient temperature on *Liothyrella neozelanica* respiration rates at 8°C (A) ( $F = 0.007$ ;  $df = 7$ ), 13°C (B) ( $F = 0.02$ ;  $df = 7$ ), 18°C (C) ( $F = 0.4$ ;  $df = 7$ ) and 23°C (D) ( $F = 0.6$ ;  $df = 7$ ).

# Appendix 2

(Seasonal biochemistry of *Liothyrella neozelanica*)

## Appendix 2.1 - Protein analysis ((Bio-Rad protein assay kit)

### Sample preparation:

1. Weigh sample dry weight (DW) (mg)
2. Grind sample to homogenise
3. Place 1-2 mg of homogenised material into a test tube
4. Add 1ml of deionised water.
5. Sonicate for 20 minutes

### Standard curve:

Diluent: Distilled water

Source: Bovine albumin (2 mg/ml)

	DILUENT (µl)	SOURCE (µl)	CONC. (µg/ml)
<b>A</b>	0	300	2000
<b>B</b>	125	375	1500
<b>C</b>	325	325	1000
<b>D</b>	175	175 from B	750
<b>E</b>	325	325 from C	500
<b>F</b>	325	325 from E	250
<b>G</b>	325	325 from F	125
<b>H</b>	400	100 from G	25
<b>I</b>	400	0	0

### Run:

6. Load 25 µl of each sample and standard into a 96-well microplate
7. Pipette 200 µl of working reagent into each well (Bio-Rad protein assay kit)
8. Incubate microplate at 37°C for 30 minutes
9. Cool microplate to room temperature
10. Measure absorbance at 562 nm

Nb. Distilled water = blank



**Appendix 2.2 – Lipid analysis** (Inouye and Lotufu 2006)Sample preparation:

1. Copy instructions 1 to 3 described in Appendix 2.1
2. Add 2 ml of chloroform / methanol (1:1)
3. Sonicate for 20 minutes
4. Centrifuge at 3000 rpm for 5 minutes
5. Transfer 250 µl of supernatant to a 13 x 100 mm test tube

Standard curve:

Diluent: Acetone

Source: Pure (100%) soybean extract (2 mg/ml)

	DILUENT (µl)	SOURCE (µl)	CONC. (µg/ml)
<b>A</b>	0	300	2000
<b>B</b>	125	375	1500
<b>C</b>	325	325	1000
<b>D</b>	175	175 from B	750
<b>E</b>	325	325 from C	500
<b>F</b>	325	325 from E	250
<b>G</b>	325	325 from F	125
<b>H</b>	400	100 from G	25
<b>I</b>	400	0	0

Run:

6. Transfer standard (250 µl) to a 13 x 100 mm test tube
7. Heat sample / standard at 100°C until solvent evaporates
8. Add 100 µl concentrated sulfuric acid and vortex.
9. Heat to 100°C for 10 minutes.
10. Cool to room temperature (20°C)
11. Add 2.4 ml of vanillin reagent and vortex
12. Transfer 225 µl to 96-well microplate
13. Read at 490nm

Nb. Acetone = blank

**Appendix 2.3 – Carbohydrate analysis (Mann and Gallagher 1985)**Sample preparation:

1. Copy instructions 1 to 3 described in Appendix 2.1
2. Add 1 ml deionised water

Standard curve:

Diluent: Distilled water

Source: Pure glucose (2 mg/ml)

	DILUENT (µl)	SOURCE (µl)	CONC.(µg/ml)
<b>A</b>	1500	500	500
<b>B</b>	1625	375	375
<b>C</b>	1750	250	250
<b>D</b>	1812.5	187.5	187.5
<b>E</b>	1875	125	125
<b>F</b>	1937.5	62.5	62.5
<b>G</b>	1968.75	31.25	31.25
<b>H</b>	1993.75	6.25	6.25
<b>I</b>	2000	0	0

Run:

3. To samples add 250 µl of chilled tri-chloroacetic acid (TCA) (15% w/v) and vortex
4. To standards add 500 µl of chilled TCA (5% w/v) and vortex
5. Cool to 4°C overnight
6. Centrifuge at 3500 rpm for 10 minutes
7. Transfer 500 µl of supernatant to a new test tube
8. Add 500 µl of deionised water and 500 µl of phenol solution (5% w/v) and vortex
9. Add 2.5 ml of concentrated sulfuric acid and vortex
10. Load 225 µl into a 96-well microplate
11. Read at 490 nm

Nb. Distilled water = blank

**Appendix 2.4 Standard curves**

For the complete set of data including standard curves, please refer to the attached CD at the back of the thesis (Appendix 5).

## Appendix 3

(Reproductive cycle of *Liothyrella neozelanica*)

### Appendix 3.1

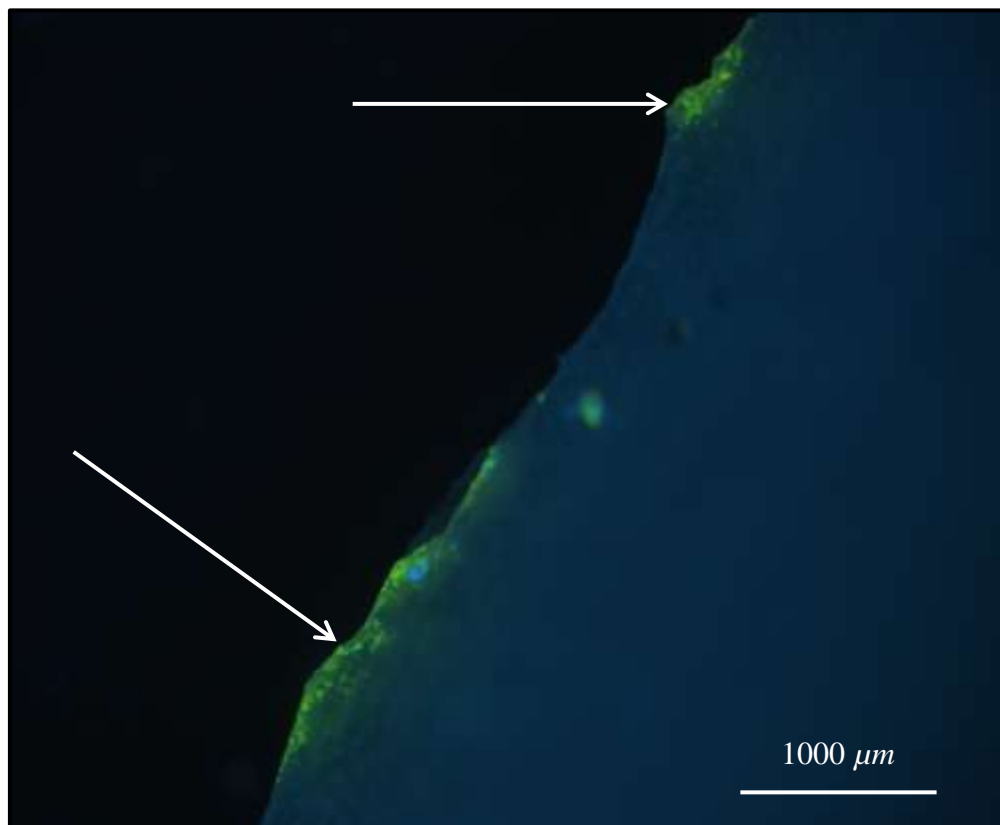


**Figure Appendix 3.1** A female *Liothyrella neozelanica* showing signs of brooding within the lophophore (White arrow).

## Appendix 4

(Annual growth of *Liothyrella neozelanica*)

### Appendix 4.1



**Figure Appendix 4.1** A ventral shell of *Liothyrella neozelanica* showing the uptake of the chemical growth marker Tetracycline under UV light (365 nm) (White arrows).

## Appendix 5

For complete data sets, please refer to the CD attached to the back page of this thesis.